

BEST AVAILABLE COPY

CERTIFICATE OF MAILING (37 CFR 1.8a)

GAN 1614

I hereby certify that this paper (along with any paper referred to as being transmitted therewith) is being deposited with the United States Postal Service on the date shown below with sufficient postage as first class mail in an envelope addressed to the: Commissioner for Patents, Washington, D.C. 20231.



Date: May 10, 2001

Dennis P. Tramaloni

(Print Name)

(Signature)

RECEIVED

MAY 10 2001

TECH CENTER 1600/2900

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Group No.: 1614

Masami Kohchi, et al.

Serial No.: 09/760,949

Filed: January 16, 2001

For: AEROTHRICIN DERIVATIVES

TRANSMITTAL OF CERTIFIED COPY

May 10, 2001

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

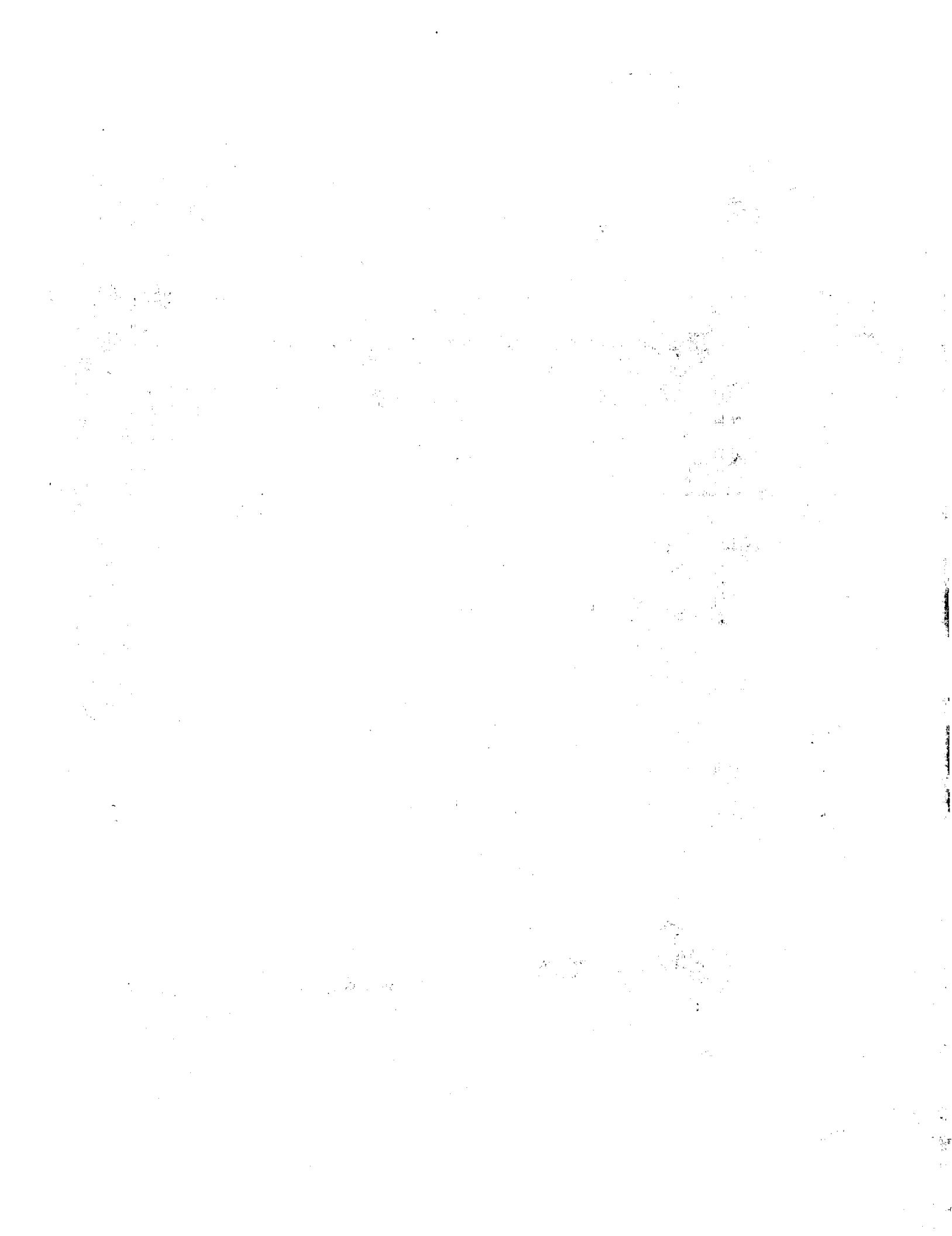
Attached please find the certified copy of the foreign application from which priority is claimed for this case:

<u>Country</u>	<u>Application No.</u>	<u>Filing Date</u>
Europe	00100807.7	January 17, 2000

Respectfully submitted,

Dennis P. Tramaloni
Attorney for Applicant
Reg. No. 28542
Hoffmann-La Roche Inc.
340 Kingsland Street
Nutley, New Jersey 07110
Phone: (973) 235-4475

DPT/lad
Enclosures
54020



09/769,949
P#3



Europäisches
Patentamt

European
Patent Office

Office européen
des brevets

Bescheinigung

Certificate

Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

00100807.7

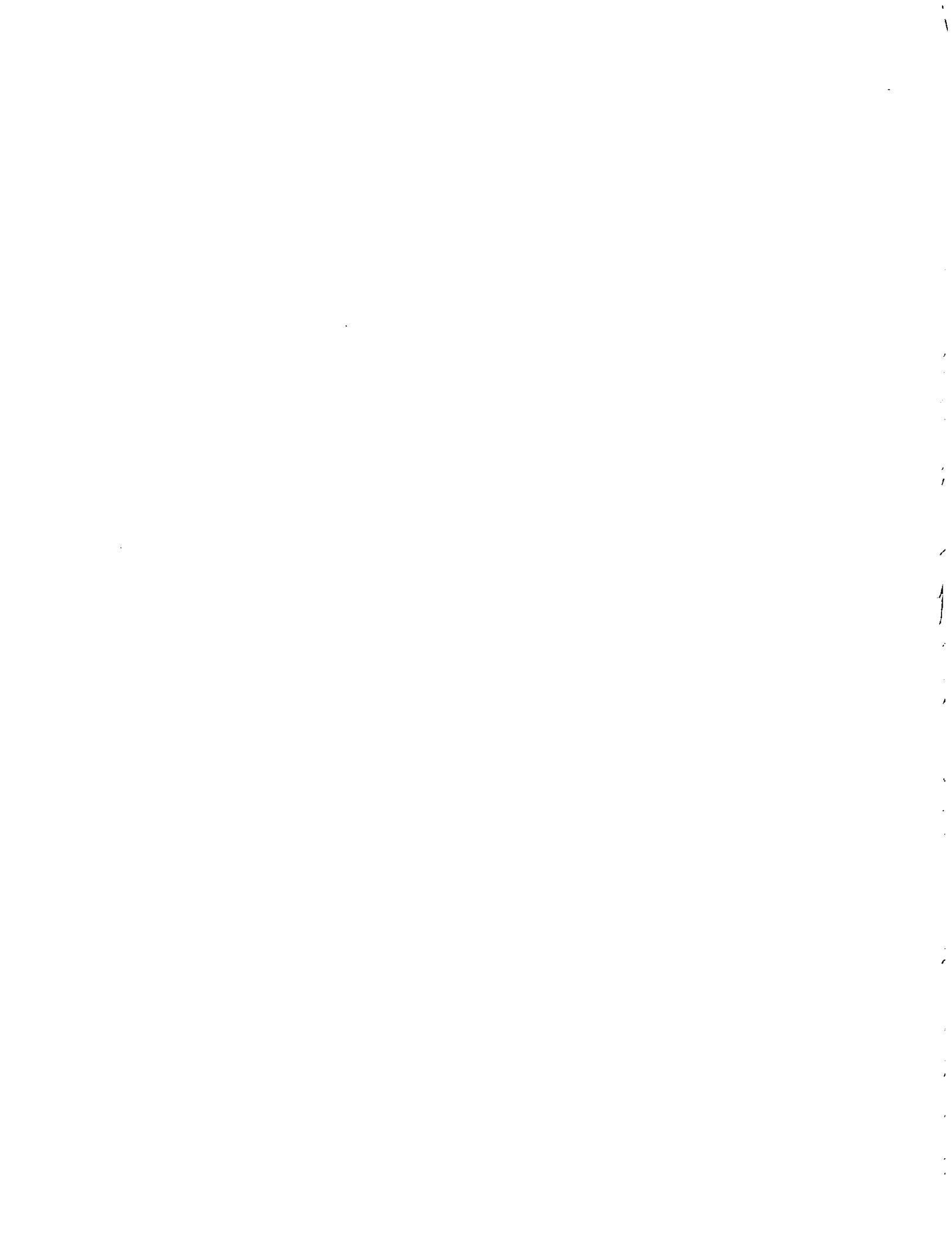
Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o:

I.L.C. HATTEN-HECKMAN

DEN HAAG, DEN
THE HAGUE, 01/11/00
LA HAYE, LE





Europäisches
Patentamt

Eur pean
Patent Office

Office européen
des brevets

Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

Anmeldung Nr.:
Application no.: **00100807.7**
Demande n°:

Anmeldetag:
Date of filing: **17/01/00**
Date de dépôt:

Anmelder:
Applicant(s):
Demandeur(s):
F. HOFFMANN-LA ROCHE AG
4070 Basel
SWITZERLAND

Bezeichnung der Erfindung:
Title of the invention:
Titre de l'invention:
Novel cyclic compounds

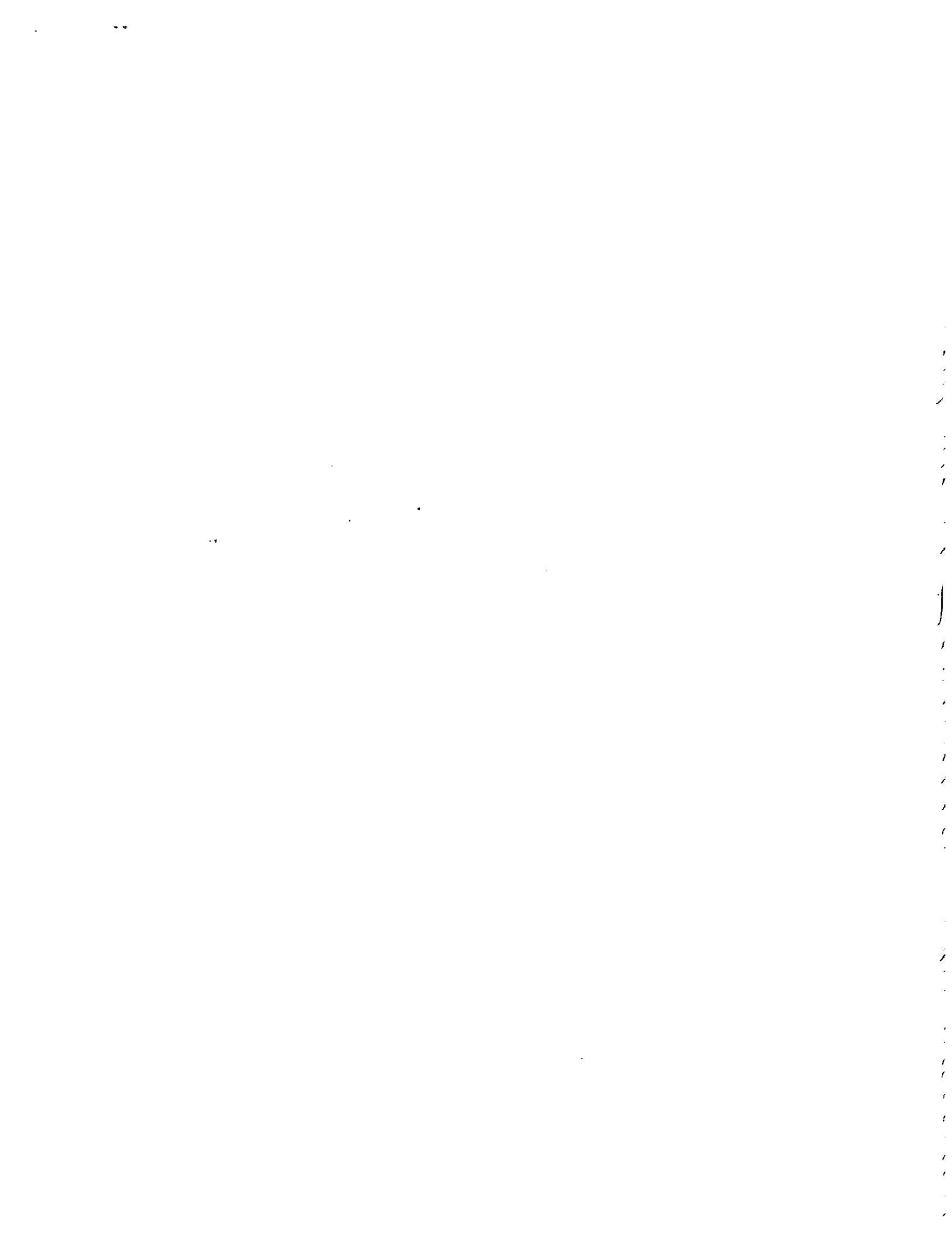
In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat:	Tag:	Aktenzeichen:
State:	Date:	File no.
Pays:	Date:	Numéro de dépôt:

Internationale Patentklassifikation:
International Patent classification:
Classification internationale des brevets:

Am Anmeldetag benannte Vertragstaaten:
Contracting states designated at date of filing: AT/BE/CH/CY/DE/DK/ES/FI/FR/GB/GR/IE/IT/LI/LU/MC/NL/PT/SE/TR
Etats contractants désignés lors du dépôt:

Bemerkungen:
Remarks:
Remarques:



F. Hoffmann-La Roche AG, CH-4070 Basle, Switzerland

EPO - Munich Case 20532
24
17. Jan. 2000

Novel Cyclic Compounds

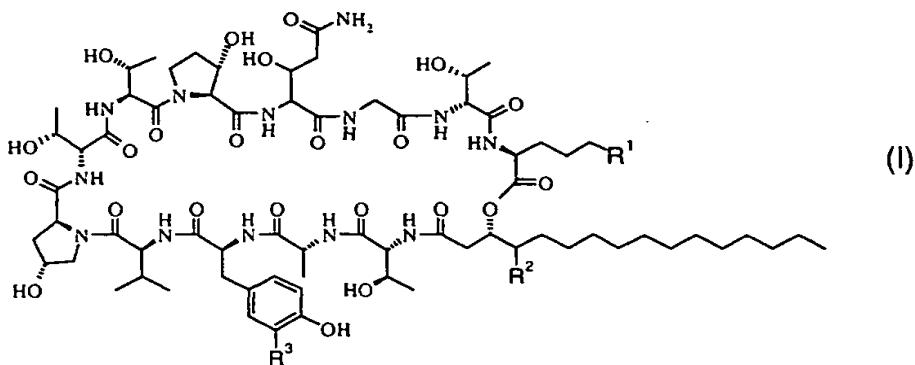
The present invention relates to novel cyclic compounds having antifungal activity (hereinafter referred to as Aerothricins), the use of Aerothricins in the medical therapy, 5 pharmaceutical compositions containing Aerothricins as well as to processes for the preparation of Aerothricins.

Azole antifungal agents are currently widely used for the treatment of systemic mycoses. However, long term prophylactic use of azole antifungals resulted in generation of azole resistant *Candida spp.* due to their fungistatic action. Therefore, fungicidal agents 10 are particularly important for treatment of severe systemic mycoses, especially against pulmonary aspergillosis. Furthermore, the currently available antifungal agents are not effective against *Scedosporium spp.* which is one of the emerging pathogens among immunocompromised patients. Amphotericin B is a highly effective fungicidal agent currently used clinically, but its therapeutic index (effective dose vs. toxic dose) is rather 15 narrow. Certain cyclic compounds such as LY303366 (EP 736 541), WF11243 (EP 584 360) are known to show fungicidal activity through inhibition of β -1,3-glucan synthase. However, they have still some disadvantages in terms of antifungal spectrum and/or safety profile. Thus, development of new fungicidal agents with better safety profile and efficacy 20 against major systemic pathogens including *Aspergillus fumigatus* and *Scedosporium spp.* is urgently required.

- 2 -

In particular, the present invention relates to novel Aerothricins represented by the Formula (I),

5



10

wherein

15

R^1 is N-(3-aminopropyl)-N-[(2S)-2,5-diaminovaleryl]amino, N-(3-aminopropyl)-N-[5-amino-2-[N,N-bis(2-aminoethyl)amino]valeryl]amino, N-(3-aminopropyl)-N-[5-amino-2-[N-(3-aminopropyl)amino]valeryl]amino, N-(2-aminoethyl)-N-[-5-amino-2-[N,N-bis(2-aminoethyl)amino]valeryl]amino or ornityl-ornitylamino;

R^2 is hydrogen or methyl;

R^3 is hydrogen or hydroxy;

and pharmaceutically acceptable salts thereof.

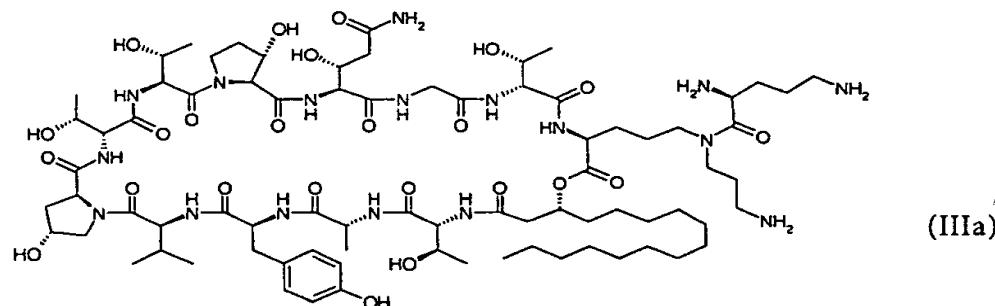
20

The present invention also relates to a pharmaceutical composition comprising an Aerothrin of Formula (I) and a pharmaceutically acceptable carrier. Furthermore, the present invention relates to the use of such Aerothricins for the preparation of medicaments, as well as to processes for the preparation of the Aerothricins of Formula (I). Additionally, the present invention relates to a method for the prophylactic and/or therapeutic treatment of infectious diseases caused by pathogenic microorganisms.

- 3 -

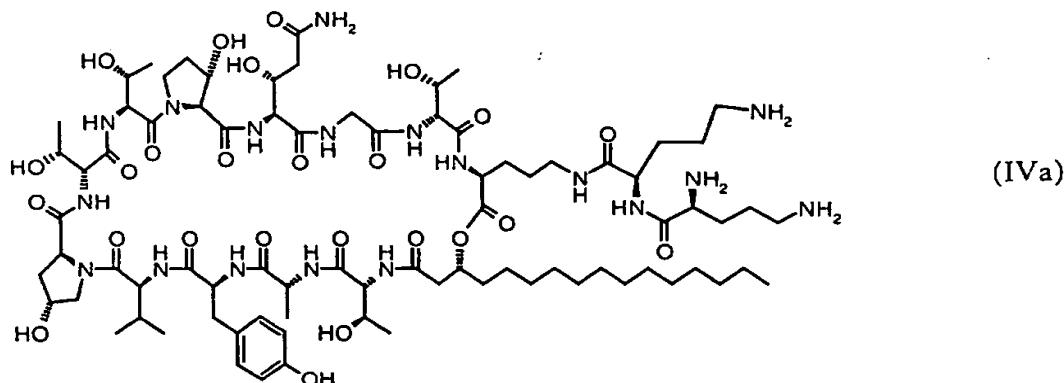
In a preferred embodiment, the present invention relates to Aerothricins of Formula (I), wherein R¹ is N-(3-aminopropyl)-N-[(2S)-2,5-diaminovaleryl]amino, N-(3-aminopropyl)-N-[(2S)-5-amino-2-[N,N-bis(2-aminoethyl)amino]valeryl]amino, N-(3-aminopropyl)-N-[(2R)-5-amino-2-[N,N-bis(2-aminoethyl)amino]valeryl]amino, N-(3-aminopropyl)-N-[(2S)-5-amino-2-[N-(3-aminopropyl)amino]valeryl]amino, N-(2-aminoethyl)-N-[(2S)-5-amino-2-[N,N-bis(2-aminoethyl)amino]valeryl]amino or (L)-ornityl-(D)-ornitylamino; R² is hydrogen or methyl, preferably hydrogen; R³ is hydrogen or hydroxy, preferably hydrogen; and pharmaceutically acceptable salts thereof.

In another preferred embodiment, the present invention relates to a compound of Formula (I), wherein R¹ is N-(3-aminopropyl)-N-[(2S)-2,5-diaminovaleryl]amino, and R² and R³ are hydrogen atoms; namely of the Formula (IIIa)



and pharmaceutically acceptable salts thereof.

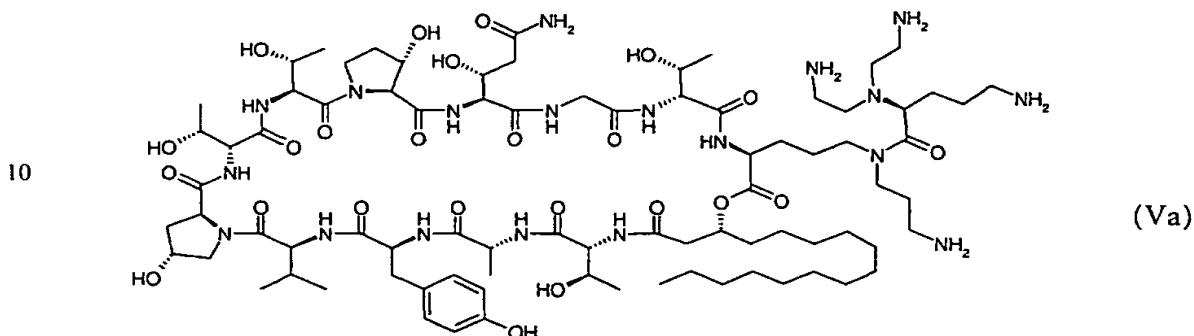
In another preferred embodiment, the present invention relates to a compound of the Formula (I), wherein R¹ is (L)-ornityl-(D)-ornitylamino, and R² and R³ are hydrogen atoms; namely of the Formula (IVa)



- 4 -

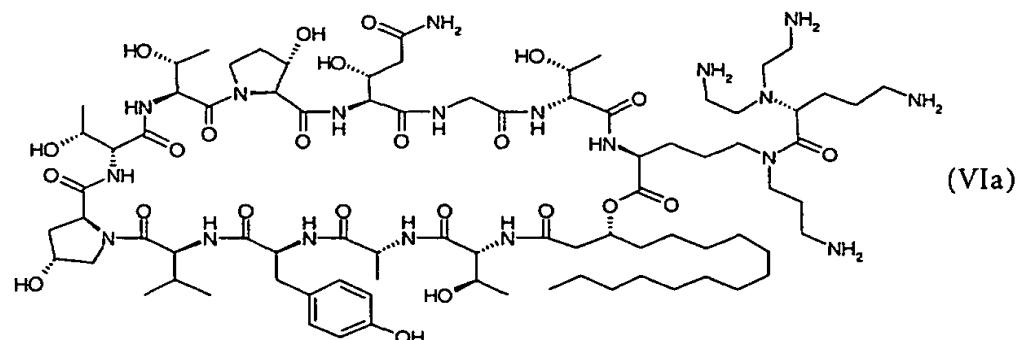
and pharmaceutically acceptable salts thereof.

In another preferred embodiment, the present invention relates to a compound of
 5 Formula (I), wherein R¹ is N-(3-aminopropyl)-N-[(2S)-5-amino-2-[N,N-bis(2-
 aminoethyl)amino]valeryl]amino, and R² and R³ are hydrogen atoms; namely of the
 Formula (Va)



and pharmaceutically acceptable salts thereof.

15 In another preferred embodiment, the present invention relates to a compound of
 Formula (I), wherein R¹ is N-(3-aminopropyl)-N-[(2R)-5-amino-2-[N,N-bis(2-
 aminoethyl)amino]valeryl]amino, and R² and R³ are hydrogen atoms; namely of the
 Formula (VIa)

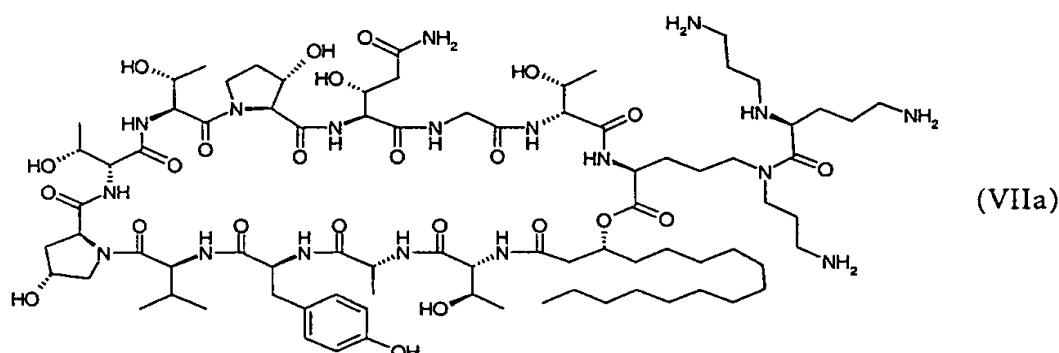


20 and pharmaceutically acceptable salts thereof.

- 5 -

In another preferred embodiment, the present invention relates to a compound of Formula (I), wherein R¹ is N-(3-aminopropyl)-N-[(2S)-5-amino-2-[N-(3-aminopropyl)amino]valeryl]amino, and R² and R³ are hydrogen atoms; namely of the
 5 Formula (VIIa)

10



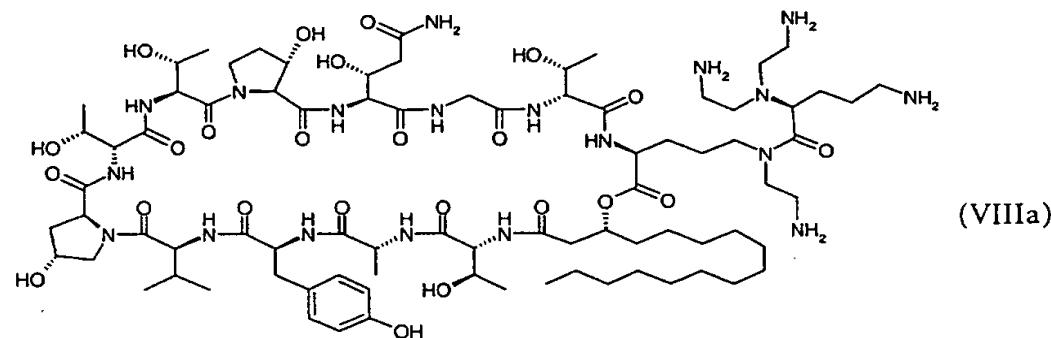
and pharmaceutically acceptable salts thereof.

15

In another preferred embodiment, the present invention relates to a compound of Formula (I), wherein R¹ is N-(2-aminoethyl)-N-[(2S)-5-amino-2-[N,N-bis(2-

15 aminoethyl)amino]valeryl]amino, and R² and R³ are hydrogen atoms; namely of the
 Formula (VIIIa)

20



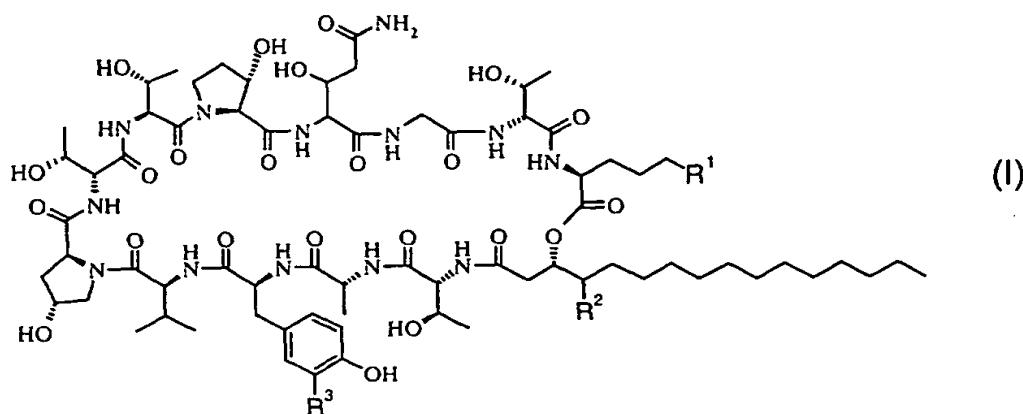
and pharmaceutically acceptable salts thereof.

25

Aerothricins in accordance with the present invention are Aerothricins exemplified
 in the following Table 1.

- 6 -

5

Table 1

Compound name	R ¹	R ²	R ³
Aerothrinic 1 (starting material)	NH ₂	CH ₃	H
Aerothrinic 2 (starting material)	NH ₂	H	OH
Aerothrinic 3 (starting material)	NH ₂	H	H
Aerothrinic 132	-NCO-CH(NH ₂)-(CH ₂) ₃ NH ₂ (CH ₂) ₃ NH ₂ **	H	H
Aerothrinic 133	-NCO-CH(H ₂) ₃ NH ₂ H * NHCOCH(CH ₂) ₃ NH ₂ NH ₂ **	H	H
Aerothrinic 134	-NCO-CH-(CH ₂) ₃ NH ₂ N[(CH ₂) ₂ NH ₂] ₂ (CH ₂) ₃ NH ₂ **	H	H
Aerothrinic 135	-NCO-CH-(CH ₂) ₃ NH ₂ N[(CH ₂) ₂ NH ₂] ₂ (CH ₂) ₃ NH ₂ *	H	H
Aerothrinic 136	-NCO-CH-(CH ₂) ₃ NH ₂ NH(CH ₂) ₃ NH ₂ (CH ₂) ₃ NH ₂ **	H	H
Aerothrinic 137	-NCO-CH-(CH ₂) ₃ NH ₂ N[(CH ₂) ₂ NH ₂] ₂ (CH ₂) ₂ NH ₂ **	H	H

* (R) configuration, ** (S) configuration

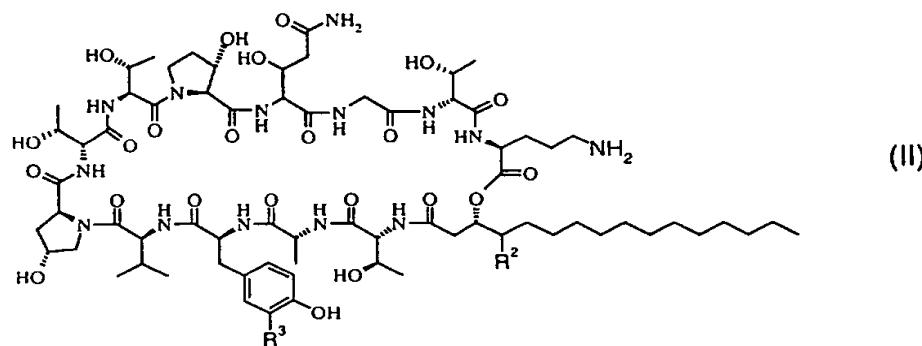
- 7 -

Aerothricins represented by Formula (I) can be produced from Aerothrin 1, 2 or 3 according to the methods outlined in scheme 1 and 2, wherein amino protecting group P₁ and P₂ can be selected from tert-butoxycarbonyl (Boc), benzyloxycarbonyl (Cbz), 5 fluorenylmethoxycarbonyl (Fmoc) and the like; R² and R³ are as defined above.

Process A

The starting compounds, Aerothricins of the Formula (II), can be produced by cultivating a microorganism belonging to *Deuteromycotina* capable of producing 10 Aerothricins 1, 2 and 3 [Aerothrin 3 (= WF11243) is described in Reference Example 1] under aerobic conditions in an aqueous or a solid medium and isolating Aerothricins 1, 2 and/or 3 from the culture.

15



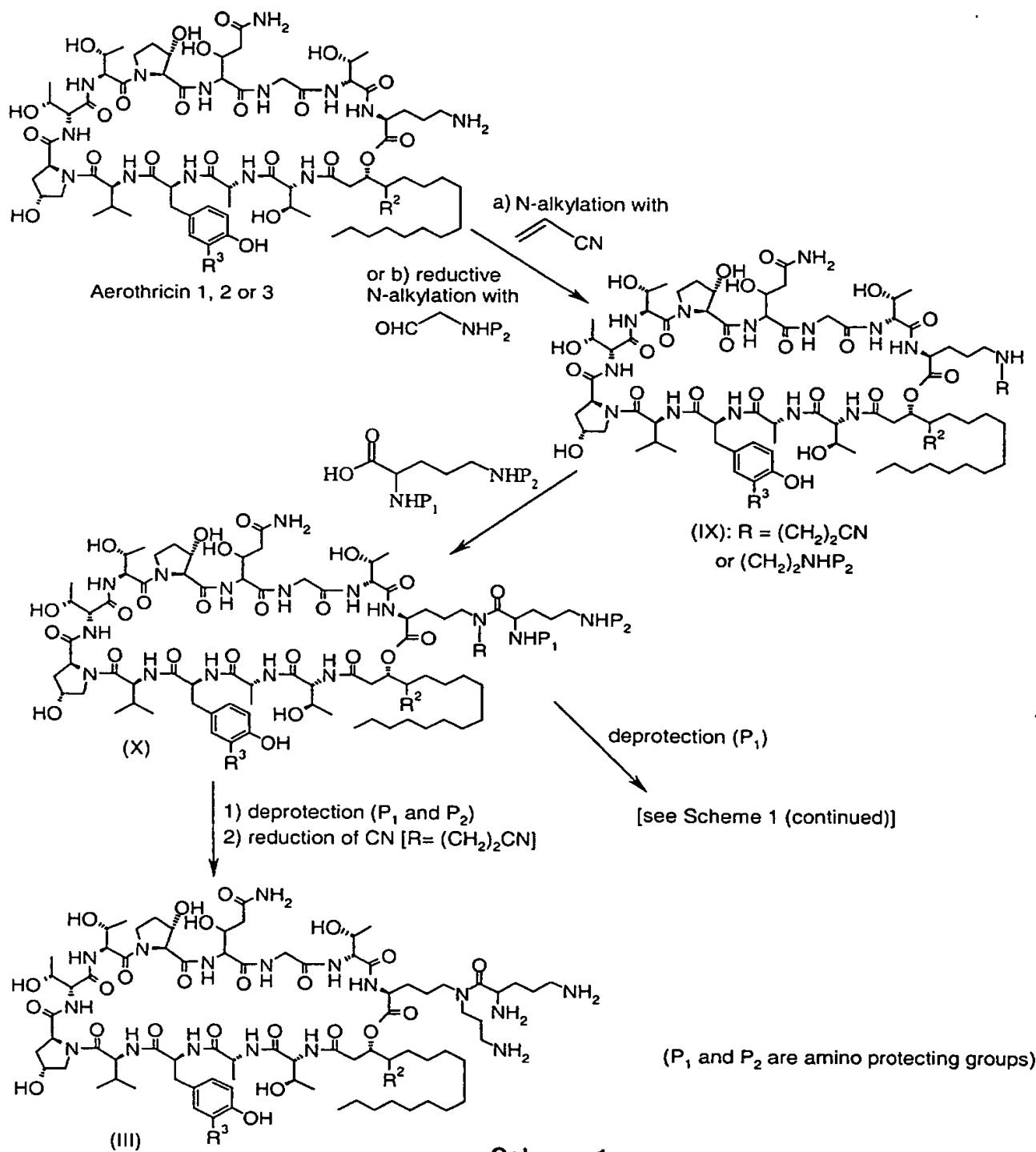
20

[wherein R² is hydrogen or methyl; R³ is hydrogen or hydroxy]

The compounds of Formula II can be converted into compounds of Formula I by the following processes B or C:

- 8 -

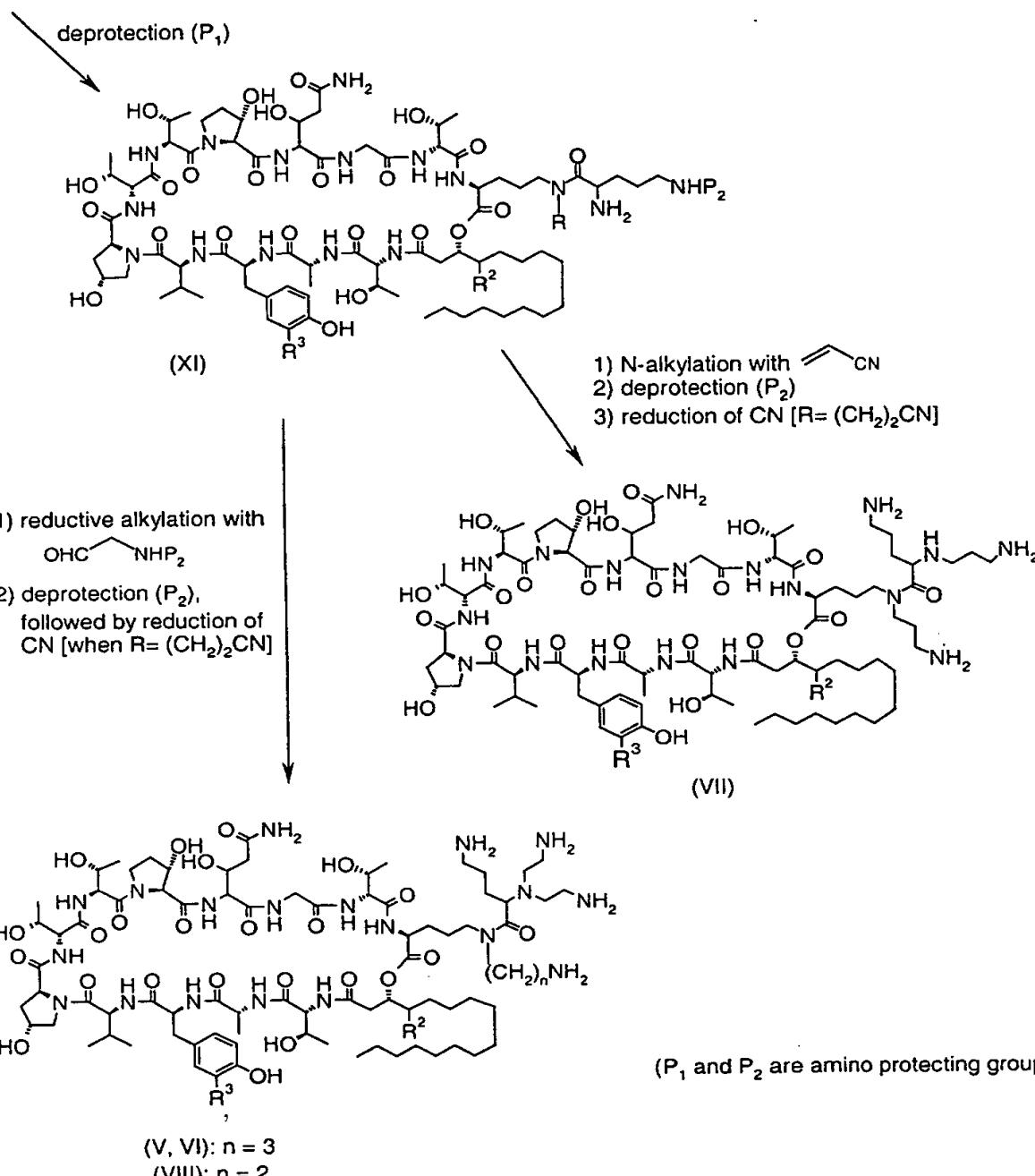
Process B



Scheme 1

- 9 -

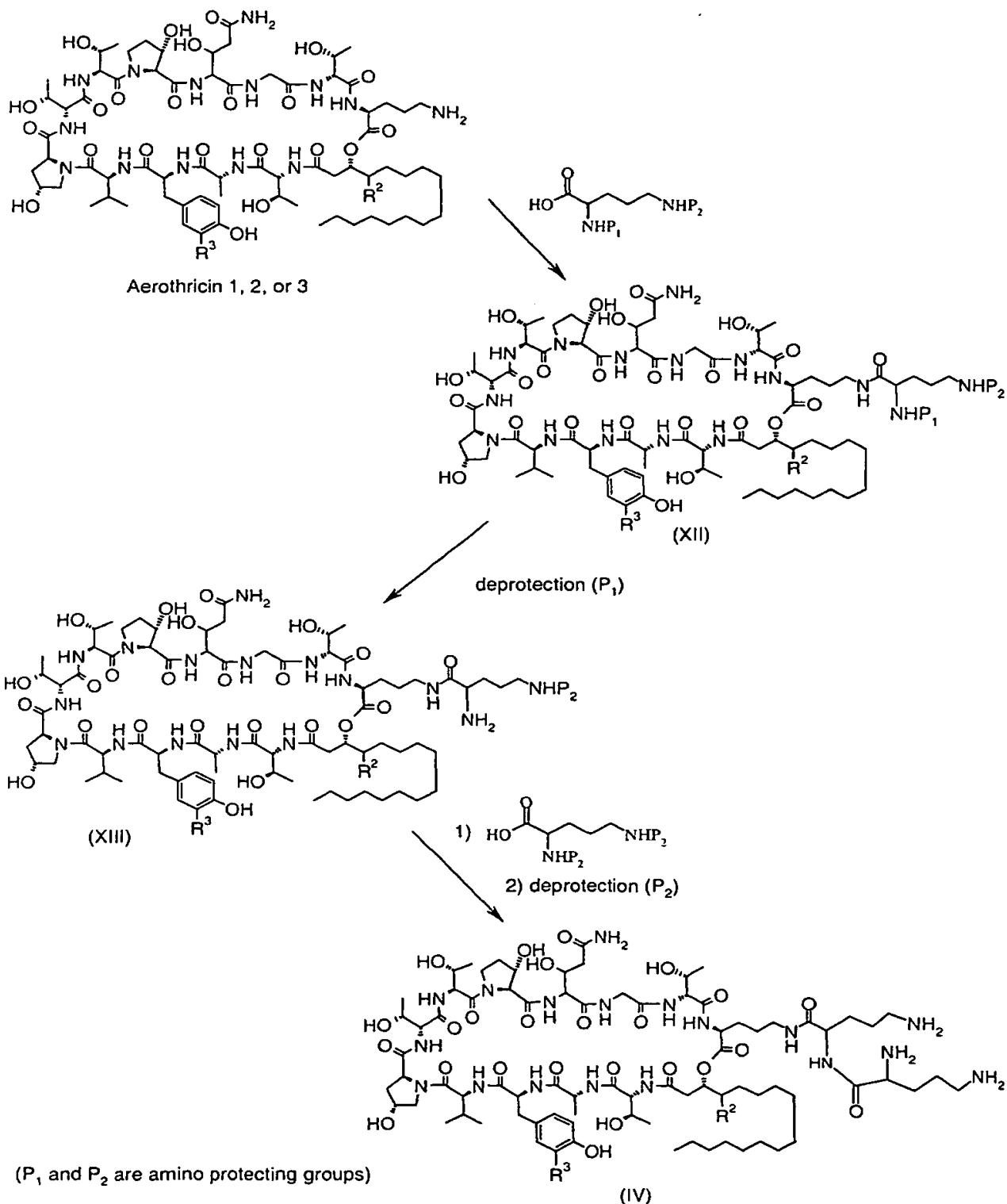
Compound (X)
[see Scheme 1]



Scheme 1 (continued)

- 10 -

Process C



Scheme 2

- 11 -

The Processes A to C can be illustrated in more detail as follows:

Process A

The microorganism used in the present invention can be any strains including mutants and variants belonging to *Deuteromycotina* capable of producing Aerothricins 1, 2 and 3. Especially preferred is strain NR 7379 which was isolated from fallen leaves collected at Kagoshima pref. in Japan, and identified as a strain belonging to *Deuteromycotina*.

The cultural and morphological characteristics of strain NR 7379 are as follows:

1. Cultural characteristics

Corn meal agar (CMA): Growth was not extensive. The colonies reached 11 mm in diameter from inoculum (4.5 mm diam. agar plug) after 14 days at 25°C. They were plane and pale cream yellow. The reverse side was pale cream yellow. Colorless and mucilaginous exudates were present.

Miura's medium (LCA): Growth was not extensive. The colonies reached 11 mm in diameter from inoculum after 14 days at 25°C. They were plane and pale cream yellow. The reverse side was pale cream yellow. Exudates were absent.

Malt extract agar (MEA): Growth was not extensive. The colonies were pustuliform and attained a diameter of 18 mm from inoculum after 14 days at 25°C. The color of colonies was light yellowish brown. The reverse side was of the same color. Exudates were colorless and mucilaginous.

Potato-dextrose agar (PDA): Growth was not extensive. The colonies were pustuliform and reached 14 mm in diameter from inoculum after 14 days at 25°C. The color and texture of colonies were similar to those on MEA. Exudates were colorless and mucilaginous.

Germination was observed between 5°C and 30°C on CMA, LCA, MEA, and PDA.

25 2. Morphological characteristics

Mycelia were partly immersed, partly superficial, branched, septate, and pale brown to cream yellow. Conidiophores were formed from immersed mycelium. They were hyaline, septate, branched, irregular. Conidiogenous cells were on distinct conidiophores or irregular hyphae. They were enteroblastic, phialidic, terminal or subterminal. Terminal or subterminal phialides were variable in length and shape. They were cylindrical to lageniform and their length and width were up to 5.5 to 10 µm and 2.5 to 5.5 µm

- 12 -

respectively. Irregularly filiform Conidiophores with lateral conidiogenous cells immediately below septa were often formed. Conidia were one-celled, hyaline, smooth, globose to subglobose, 2.0 to 5.5 μm in length and 2.0 to 5.0 μm in width.

On the basis of these distinct cultural and morphological characteristics, the present
5 strain belonged to *Deuteromycotina* designated as Deuteromycotina NR 7379.

The strain denoted as Deuteromycotina NR 7379 has been deposited with the
National Institute of Bioscience and Human-Technology, Agency of Industrial Science and
Technology, Japan in the name of Nippon Roche K.K., of 6-1, Shiba 2-chome, Minato-ku
Tokyo, 105 Japan on June 16, 1998 under the Budapest Treaty as follows:

10 Deuteromycotina NR 7379 (FERM BP-6391).

The cultivation in accordance with the process provided by the present invention
can be carried out in a culture medium which contains customary nutrients usable by the
microorganism being cultivated. As carbon sources there can be mentioned, for example,
glucose, sucrose, starch, glycerol, molasses, dextrin and mixtures thereof. Nitrogen sources
15 are, for example, soybean meal, cottonseed meal, meat extract, peptone, dried yeast, yeast
extract, corn steep liquor, ammonium sulfate, sodium nitrate and mixtures thereof.
Moreover, there may be added to the culture medium other organic or inorganic
substances for promoting the growth of the microorganism and for increasing the
20 production of Aerothrinic 1. Examples of such substances are inorganic salts, such as
calcium carbonate, sodium chloride, phosphates and the like.

The cultivation is carried out under aerobic conditions preferably in a liquid
medium by submerged fermentation, or in a solid medium by static fermentation. A
temperature of 20°C to 30°C, with an optimal temperature of 27°C is suitable for
cultivation. The cultivation is preferably carried out at a pH of 3 to 9. The cultivation time
25 depends on the conditions under which the cultivation is carried out. In general, it is
sufficient to carry out the cultivation for 20 to 360 h.

For harvesting the objective Aerothricins 1, 2 and 3 from the cultures, separation
methods which are usually employed to isolate metabolites produced by microbes from
30 their cultures can be properly used. For example, Aerothrinic 1, which is a methanol
extractable amphoteric substance, is recovered advantageously by the following
procedures.

That is, the whole culture solid obtained by solid state fermentation is extracted with
an appropriate solvent to recover the proposed product. The solvents which can be used to

- 13 -

extract the objective compound from the whole cultured solid include water-soluble organic solvents or hydrous solutions of water-soluble organic solvents, such as methanol, ethanol and hydrous alcohols.

For removing salts, water soluble substances, etc. from the resulting extract, use is made of, with advantage, solvent partition between water and water-immiscible organic solvents, such as *n*-butanol, ethyl acetate, etc. For removing coloring substances, fat-soluble substance or the like from the extract, use is made of, with advantage, solvent purification by methanol, ethanol, a mixture of acetonitrile-0.1% aqueous trifluoroacetic acid, etc.

For complete purification of Aerothricins, column chromatography is used with advantage. Carriers which can be used in such a column chromatography are such as YMC-GEL ODS (Yamamura Chemical Laboratories, Japan) or Preparative C18 (Waters Millipore Corporation). As an eluent, use is made of a solvent system consisting a mixture of aqueous trifluoroacetic acid and appropriate water-soluble organic solvents such as methanol, ethanol, acetonitrile, etc. The eluate fraction thus purified, which contains each component, can be subjected to concentration or freeze-drying to pulverize Aerothricins 1, 2 and 3.

Aerothricins 1, 2 and 3 were isolated as a trifluoroacetic acid salt, but the free Aerothricins 1, 2 and 3 can be prepared by the following procedure. Namely, Aerothricins 1, 2 and 3 trifluoroacetic acid salt are dissolved in water, to which was added one equivalent of sodium hydroxide, and the mixture is subjected to Sephadex LH-20 column Chromatography, followed by elution with a hydrous alcohol such as methanol-water, etc. to thereby obtain Aerothricins 1, 2 and 3 (free form), respectively.

Process B

The compound of Formula (III) can be prepared from Aerothricins of Formula (II) [wherein R¹ is an amino group; R² and R³ are as defined above] in 4 steps: (1) N-alkylation with acrylonitrile, (2) acylation with N-protected ornitine, (3) removal of amino protecting groups (P₁ and P₂), and (4) reduction of cyano group.

The compound of Formula (V, VI) can be prepared from Aerothricins of Formula (II) [wherein R¹ is an amino group; R² and R³ are as defined above] in 6 steps: (1) N-alkylation with acrylonitrile, (2) acylation with N-protected ornitine, (3) removal of amino protecting group (P₁), (4) reductive N-alkylation with N-protected amino-acetaldehyde, (5) removal of amino protecting group (P₂), and (6) reduction of cyano group.

- 14 -

The compound of Formula (VIII) can be prepared from Aerothricins of Formula (II) [wherein R¹ is an amino group; R² and R³ are as defined above] in 6 steps: (1) reductive N-alkylation with N-protected amino-acetaldehyde, (2) acylation with N-protected ornitine, (3) removal of amino protecting group (P₁), (4) reductive N-alkylation with N-protected amino-acetaldehyde, (5) removal of amino protecting group (P₁), and (6) reduction of cyano group.

The compound of Formula (VII) can be prepared from Aerothricins of Formula (II) [wherein R¹ is an amino group; R² and R³ are as defined above] in 6 steps: (1) N-alkylation with acrylonitrile, (2) acylation with N-protected ornitine, (3) removal of amino protecting group (P₁), (4) N-alkylation with acrylonitrile, (5) removal of amino protecting group (P₂), and (6) reduction of cyano group.

Process C

The compound of Formula (IV) can be prepared from Aerothricins of Formula (II) [wherein R¹ is an amino group; R² and R³ are as defined above] in 4 steps: (1) acylation with N-protected ornitine, (2) removal of amino protecting group (P₁), (3) acylation with N-protected ornitine, and (4) removal of amino protecting group (P₂).

In the above processes B and C;

(a) N-monoalkylation of an amino group can be done using acrylonitrile according to the method described in Organic Synthesis col. Vol. III, page 93.

(b) N-alkylation of the primary or secondary amino group can be done by the conventional reductive alkylation with N-protected aminoacetaldehyde using a reducing agent such as sodium cyanoborohydride in the presence or absence of weak acid such as acetic acid. The reaction can be carried out at room temperature in a solvent such as methanol, ethanol, acetic acid and the like.

(c) The reduction of the nitrile group can be achieved by catalytic hydrogenation or reduction with sodium borohydride/cobalt chloride, borane-methylsulphide complex and the like [cf. J. Med. Chem., 37, 222 (1994)].

(d) N-acylation of an amino group can be done with N-protected ornitine using condensation agents such as dicyclohexylcarbodiimide, BOP, HBTU, TNTU, PyBOPTM, PyBOPTM, TBTU, TSTU, HOBT and the like, or the combination of two of them. The reaction can be carried out in a solvent such as methanol, ethanol, pyridine, N,N-dimethylformamide, N-methylpyrrolidone and the like in the presence or absence of a

- 15 -

base such as triethylamine, di-isopropylethylamine, pyridine and the like at a temperature between -20°C and +50°C, preferably at 0°C to +25°C.

(e) The removal of the amino protecting group can be done by the method known to those skilled in the art, e.g. treatment with trifluoroacetic acid for Boc group, or piperidine 5 for Fmoc group.

The present invention is also concerned with acid addition salts of Aerothricins. The acid addition salt can be obtained as trifluoroacetic acid salt after normal course of isolation. The salt thus obtained may be dissolved in water and passed through an anion 10 exchange column bearing the desired anion. The eluate containing the desired salt may be concentrated to recover the salt as a solid product.

The Aerothricins of Formula (I) may be converted to a corresponding salt by virtue of the presence of the tertiary nitrogen atoms.

The acid addition salt of Aerothricins of Formula (I) can be obtained by treatment of 15 the free base of Aerothricins with at least a stoichiometric amount of an appropriate acid, such as mineral acids, e.g., hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, and the like, and organic acids, e.g., acetic acid, propionic acid, glycolic acid, pyruvic acid, oxalic acid, malic acid, malonic acid, succinic acid, maleic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, methanesulfonic 20 acid, ethanesulfonic acid, p-toluenesulfonic acid, salicylic acid, and the like. Typically, the free base is dissolved in an inert organic solvent such as ethanol, methanol, and the like, and the acid added in a similar solvent. The temperature is maintained at about 40°C. The resulting salt precipitates spontaneously or may be brought out of solution with a less polar solvent.

25 The acid addition salts of the Aerothricins of Formula (I) may be converted to the corresponding free base by treatment with at least a stoichiometric amount of a suitable base such as sodium or potassium hydroxide, potassium carbonate, sodium bicarbonate, ammonia, and the like.

Aerothricins provided by the present invention exhibit broad fungicidal activity 30 against various fungi and can be used as agents for treatment and prophylaxis of fungal infectious diseases. The *in vitro* and *in vivo* antifungal activity (see Tables 2, 3-1 and 3-2) as well as the toxicity to hepatocytes (see Table 4) of the representative Aerothricins of Formula (I) are shown as follows:

- 16 -

1. In vitro antifungal activities

The *in vitro* antifungal activities of the representative Aerothricins of the present study were evaluated by determining the 80% inhibitory concentration (IC_{80}), which was calculated as the lowest concentration of an antifungal to inhibit the growth of fungus to 5 20% turbidity compared with the drug-free control growth spectrophotometrically.

The IC_{80} values were determined by the broth micro-dilution procedure based on NCCLS Approved Standard with the following minor modifications (National Committee for Clinical Laboratory Standards. (1997) Reference method for broth dilution antifungal susceptibility testing for yeasts. Approved standard. Document M27-A). Yeast Nitrogen 10 Base (YNB; Difco Lab.) supplemented with 1% glucose and 0.25% K_2HPO_4 was used as testing medium for yeasts, the same medium solidified with 0.2% low melting point agarose (BRL) was used for filamentous fungi. Inoculum size was $1-3 \times 10^3$ cells/ml, and incubation was performed for 1-2 days at 35°C.

15 Table 2: *In vitro* Antifungal activity, IC_{80} ($\mu\text{g}/\text{ml}$)

	<i>Candida albicans</i> CY1002	<i>Aspergillus fumigatus</i> CF1003	<i>Scedosporium apiospermum</i> CF1077
Aerothrinicin 132	0.28	2.9	0.38
Aerothrinicin 134	0.37	0.58	0.37
Aerothrinicin 135	0.41	0.37	0.35
Aerothrinicin 136	0.33	0.38	0.34

2. In vivo antifungal efficacy

2-1: Murine systemic candidiasis

In vivo antifungal efficacy of Aerothricins of the present invention against systemic candidiasis is shown in the following Table 3-1. Mice of a conventional immunocompetent mouse strain, Crj: CD-1 (ICR) were used for experimental infection models of systemic candidiasis. 4 weeks old Crj: CD-1 (ICR) mice were used for systemic candidiasis by injecting *Candida albicans* 5×10^6 conidia/mouse via the tail vein. Test compounds were intravenously (i.v.) given once just after infection for systemic candidiasis. 50% of effective 20 dose (ED_{50}) values were calculated from the survival number at each dose on day 7. 25

- 17 -

Table 3-1: *In vivo* antifungal activity against systemic candidiasis in mice,
ED₅₀ (mg/kg) on day 7

Aerothrinicin 132	0.43
Aerothrinicin 133	0.35
Aerothrinicin 135	0.35

2-2: Murine pulmonary aspergillosis

5 *In vivo* antifungal efficacy of Aerothricins of the present invention against pulmonary aspergillosis is shown in the following Table 3-2. Murine pulmonary aspergillosis was created in cortisone-treated (250mg/kg, twice sub-cutaneous treatments on 3 days before and on the infection day) ICR male mouse. Conidia of *A.fumigatus* (2.5×10^5 conidia/mouse) were infected intratracheally to these mice, and treatments were carried
10 out once daily for 4 days. The efficacy of each drug was determined from the survival number, and 50% of effective dose (ED₅₀) was calculated from the survival number at each dose on the 14 days.

Table 3-2: *In vivo* antifungal activity against pulmonary aspergillosis in mice,
ED₅₀ (mg/kg) on day 14

Aerothrinicin 132	5.2
Aerothrinicin 134	5.8
Aerothrinicin 137	5.2
Aerothrinicin 3	>15

15

3. *In vitro* hepatotoxicity test

The mouse hepatocytes were isolated by a collagenase digestion and cultured in microtest plates. The hepatocyte monolayers were exposed to the test Aerothricins in the culture system for 1 day. After the culture period, the hepatocytes were observed under a
20 microscope and evaluated morphologically. The degree of the morphological alteration (degeneration) of the hepatocytes by the test Aerothricins were compared with WF11243 and LY303366.

- 18 -

Table 4: Cytotoxicity to hepatocyte ($\mu\text{g}/\text{ml}$)

Aerothrinic 132	>100
Aerothrinic 134	>100
Aerothrinic 135	>100
WF11243 (= Aerothrinic 3)	100
LY303366	10

5 mg/kg and 30 mg/kg of Aerothrinic 132 administration (once daily: i.v.) to mice for 2 weeks showed no acute toxicity.

5

Therefore, the novel Aerothricins of Formula (I) as well as pharmaceutically acceptable salts thereof exhibit potent antifungal activity against various fungal infections, including Aspergillosis, in mice over a wide range of dosages and are useful as antifungal agents. Moreover, the Aerothricins provided by this invention are much less cytotoxic to 10 hepatocytes than the known cyclic peptide derivatives (WF11243 and LY303366).

Aerothricins of the present invention may also be useful for inhibiting or alleviating *Pneumocystis carinii* infections in immune-compromised patients.

The present invention further relates to the pharmaceutical compositions containing the novel Aerothricins of Formula (I) as well as pharmaceutically acceptable salts thereof.

15 The novel Aerothricins of Formula (I) as well as pharmaceutically acceptable salts thereof are highly active fungicidal agents. They are active against a variety of fungal species including *Candida spp.*, *Aspergillus spp.*, *Scedosporium spp.*, *Mucor spp.* and *Absidia spp.*.

Thus, Aerothricins of the present invention are useful for topical and systemic 20 treatment of mycoses in animals as well as humans. For example, they are useful in treating topical and mucosal fungal infections caused by *Candida spp.*, *Trichophyton spp.*, and *Microsporum spp.*. They may also be used in the treatment of systemic fungal infections caused by, for example, *Candida spp.*, *Aspergillus spp.*, or *Scedosporium spp.*.

For clinical use, the novel Aerothricins of Formula (I) as well as pharmaceutically 25 acceptable salts thereof can be administered alone, but will generally be administered in pharmaceutical admixture formulated as appropriate to the particular use and purpose desired, by mixing excipient, binding agent, lubricant, disintegrating agent, coating

- 19 -

material, emulsifier, suspending agent, solvent, stabilizer, absorption enhancer and/or ointment base. The admixture can be used for oral, injectable, nasal, rectal or topical administration.

Pharmaceutical formulations of Aerothricins for oral administration may be granule,
5 tablet, sugar coated tablet, capsule, pill, suspension or emulsion. For parenteral injection,
for example, intravenously, intramuscularly or subcutaneously, Aerothricins of Formula
(I) may be used in the form of a sterile aqueous solution which may contain other
substances, for example, salts or glucose to make the solution isotonic. These compositions
also may be presented in unit dosage form in ampoules or in multidose containers,
10 preferable with added preservatives. Alternatively, the active ingredients may be in powder
form for reconstituting with a suitable vehicle prior to administration. Aerothricins can
also be administered in the form of a suppository or pessary, or they may be applied
topically in the form of a lotion, solution, cream, ointment or dusting powder.

The daily dosage level of Aerothricins of Formula (I) is from 0.1 to 50 mg/kg (in
15 divided doses) when administered by either the oral or parenteral route. Thus tablets or
capsules of Aerothricins can be expected to contain from 5 mg to 0.5 g of active compound
for administration singly or two or more at a time as appropriate. In any event the actual
dosage can be determined by the physician and it may be varied upon the age, weight and
response of the particular patient.

20 When Aerothricins are for antifungal use any method of administration may be
employed. For treating mycotic infections, oral or intravenous administration is usually
employed.

When Aerothricins are to be employed for control of pneumocystis infections it is
desirable to directly treat lung and bronchi. For this reason inhalation methods are
25 preferred. For administration by inhalation or nasal, Aerothricins of the present invention
are conveniently delivered in the form of an aerosol spray presentation from pressurized
packs or nebulisers. The preferred delivery system for inhalation or nasal is a metered dose
inhalation aerosol, which may be formulated as a powder, suspension or solution of a
compound of Formula (I) in suitable propellants, such as fluorocarbons or hydrocarbons.

30 Although Aerothricins of the present invention may be employed as tablets, capsules,
topical compositions, insufflation powders, suppositories, and the like, the solubility of
Aerothricins of the present invention in water and aqueous media render them adaptable
for use in injectable formulations and also in liquid compositions suitable for aerosol
sprays.

- 20 -

The following Examples illustrate the preferred methods for the preparation of Aerothricins of the present invention, which are not intended to limit the scope of the invention thereto.

In the following Examples, the products were analyzed and purified by HPLC using a
5 reverse phase column selected from those listed below. The mixed solvent consisted of 0.05% trifluoroacetic acid-water : 0.05% trifluoroacetic acid-acetonitrile with the appropriate ratio described in each working Example.

HPLC columns:

	Column A:	CAPCELL PAK C18, UG-120, 4.6x250mm
10	Column B:	CAPCELL PAK C18, UG-120, 10x250mm
	Column C:	CAPCELL PAK C18, UG-80, 20x250mm
	Column D:	CAPCELL PAK C18, SG-120, 4.6x250mm
	Column E:	CAPCELL PAK C18, SG-120, 10x250mm
	Column F:	TSK GEL ODS-80Ts, 20x250mm

15

- 21 -

In the following working Examples, Aerothricins were obtained as trifluoroacetic acid salts unless otherwise indicated.

Reference Example 1

5 Preparation of Aerothricins 1, 2 and 3

a) Solid fermentation

A 0.1 ml portion of the frozen culture of Deuteromycotina NR 7379 (FERM BP-6391) in 10% (v/v) glycerol solution was defrosted and inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a medium consisting of 2% glucose, 1% potato starch, 1.5% glycerol, 1% Toast soya (Nissin Seiyu), 0.35% yeast extract (Nippon Seiyaku), 0.25% Polypepton (Nihon Seiyaku), 0.3% NaCl, 0.5% CaCO₃, 0.005% ZnSO₄·7H₂O, 0.0005% CuSO₄·5H₂O, and 0.0005% MnSO₄·4H₂O. The pH of the medium was not adjusted. The seed culture was incubated on a rotary shaker at 27°C for 7 days at 220 rpm. 2 ml of the seed culture was transferred into a 3-liter Erlenmeyer flask containing a solid medium consisting of 200 g pressed barley, 0.12 g yeast extract (Difco), 0.06 g sodium tartarate, 0.06 g KH₂PO₄, and 120 ml water. The fermentation was carried out at 27°C under static condition. The production reached maximum at around 240 h of fermentation and the culture was subjected to the isolation procedure of Aerothricins 1, 2 and 3.

20

The cultured solid (10 kg) obtained was added methanol (40 L) and the mixture was stirred, followed by removal filtration to obtain methanol extract (39 L). The methanol extract thus obtained was concentrated to dryness under reduced pressure, and the residue (64.8 g) was added ethyl acetate (1 L) and water (1 L). And the mixture was stirred, followed by removal of the ethyl acetate layer.

Furthermore, the aqueous layer was likewise washed with ethyl acetate (1 L) twice. The remaining aqueous layer was extracted with *n*-butanol (1 L) three times. The extracts thus obtained were combined and concentrated to dryness under reduced pressure, and 30 the residue (28.5 g) was dissolved into a mixture (250 ml) of acetonitrile-0.1% aqueous trifluoroacetic acid (1:1). After removal of the insoluble materials by centrifugation, the solution thus obtained was evaporated to dryness under reduced pressure, and the residue was added methanol (300 ml) and the mixture was stirred, followed by removal filtration

- 22 -

to obtain the methanol solution (280 ml). The methanol soluble materials (9.3 g) thus obtained were then subjected to a column chromatography on reversed phase silica gel C18 (1 L). The column was eluted stepwise using a mixture of methanol-0.1% aqueous trifluoroacetic acid (2:8, 4:6, 5:5, 6:4, 7:3, and 8:2). The Aerothricins 1, 2 and 3 eluted in
5 this order with methanol-0.1% aqueous trifluoroacetic acid (7:3) were concentrated to dryness *in vacuo* to obtain white powdery Aerothrinic 3 trifluoroacetic acid salt (731 mg) and Aerothrinic 1 trifluoroacetic acid salt (747 mg), respectively. The fractions containing Aerothrinic 2 was concentrated under reduced pressure and further purified by HPLC
10 under the following conditions: column: Capcell Pak C18 (i.d. 30 x 250 mm, Shiseido Co., LTD.); mobile phase: acetonitrile-0.1% aqueous trifluoroacetic acid (45:55); flow rate: 40 ml/min.; detection: UV 220 nm. The appropriate eluates obtained under the above conditions were concentrated to dryness *in vacuo* to obtain white powdery Aerothrinic 2 trifluoroacetic acid salt (42 mg).

15 b) Flask fermentation

A 2 ml portion of the frozen culture of Deuteromycotina NR 7379 (FERM BP-6391) in 10% (v/v) glycerol solution was defrosted and inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a medium consisting of 1% glucose, 1% oat flour, 4% tomato paste, 0.5% corn steep liquor (Ando kasei), 0.001% FeSO₄·7H₂O, 0.001% MnSO₄·4H₂O, 0.0001%
20 CaCl₂, 0.0002% ZnSO₄·7H₂O, 0.00002% (NH₄)₆MoO₂·4H₂O, and 0.00006% H₃BO₃. The pH of the medium was adjusted to 6.8 before sterilization. The seed culture was incubated on a rotary shaker at 27°C for 3 days at 220 rpm. 2 ml of the first seed culture was transferred into 500-ml Erlenmeyer flasks containing 100 ml of the same medium and incubated on a rotary shaker under the same conditions for 3 days. 2 ml of the second seed
25 culture was inoculated into 500-ml Erlenmeyer flasks containing 100 ml of the medium consisting of 8.5% glycerol, 1% pectin from citrus, 0.4% peanuts powder, 0.4% casein from milk vitamin-free, 0.4% tomato paste, 0.4% corn steep liquor (Ando kasei), 0.2% glycine, and 0.2% KH₂PO₄. The pH of the medium was adjusted to 7.0 before sterilization. The fermentation was conducted at 27°C with agitation of 220 rpm. After 10 days
30 cultivation, the production reached maximum and the whole culture was subjected to the isolation procedure of Aerothricins 1, 2 and 3.

c) Jar fermentation

A 2 ml portion of the frozen culture of Deuteromycotina NR 7379 (FERM BP-6391) in 10% (v/v) glycerol solution was defrosted and inoculated into a 500-ml Erlenmeyer flask

- 23 -

containing 100 ml of the same seed medium as described above. The flask was shaken at 220 rpm for 3 days at 27°C. 2 ml of the first seed culture was transferred into 500-ml Erlenmeyer flasks containing 100 ml of the same seed medium and incubated on a rotary shaker under the same conditions for 3 days. Six hundred ml of the second seed culture 5 was inoculated into 50-liter jar fermentor containing 30 liters of the same production medium as described above and 0.4% disfoam (Nissan Disfoam CA-123). The fermentation was carried out at 27°C, with aeration of 30 liters/min. and agitation of 400 rpm. The production reached maximum at around 168 h of fermentation and the whole culture was subjected to the isolation procedure of Aerothricins 1, 2 and 3.

10

Aerothrinic 1

- 1) Appearance:
white solid
- 2) Molecular weight (FAB-MS method):
15 m/z 1547 ($M+H$)⁺
- 3) Molecular formula:
 $C_{72}H_{118}N_{14}O_{23}$
- 4) High resolution mass spectroscopy (for $M+H$)⁺:
Found: 1547.8568
Calculated for $C_{72}H_{119}N_{14}O_{23}$: 1547.8572
- 5) UV spectrum (Fig. 1): in methanol:
 $\lambda(\epsilon)_{\text{max}}$ (in MeOH): 225±5 (10600 sh), 270±5 (2000), 278±5 (2100)
 $\lambda(\epsilon)_{\text{max}}$ (in N/10 NaOH-MeOH): 240±5 (7700), 268±5 (1800), 298±5 (1800)
- 6) IR spectrum (KBr) (Fig. 2):
25 Main absorption wave numbers (cm^{-1}) are as follows:
3379, 2927, 2855, 1740, 1660, 1535, 1453, 1203, 1139, 837
- 7) $^1\text{H-NMR}$ spectrum (Fig. 3):
400 MHz, in CD_3OD
- 8) $^{13}\text{C-NMR}$ spectrum (Fig. 4):
30 100 MHz, in CD_3OD
- 9) Solubility:
Soluble: water, methanol, dimethylsulfoxide

- 24 -

10) Color reaction:

Positive: ninhydrin, anisaldehyde-sulfuric acid, iodine vapor, vanillin-sulfuric acid,
Rydon-Smith reagent, molybdophosphoric acid

Negative: Sakaguchi reagent, Bromocresol green, 2,4-dinitrophenylhydrazine-sulfuric acid

5

11) Thin-layer chromatography (TLC):

Carrier	Solvent	Rf
silica gel F254 ¹	n-BuOH: acetone:AcOH:H ₂ O (4:5:1:1)	0.74
	MeOH: H ₂ O (95:5)	0.12

10 ¹ E. Merck AG., Germany

12) High Performance Liquid Chromatography:

Carrier: Capcell Pak C18 gel S120A, 4.6x250 mm (manufactured by Shiseido, Co., LTD.)

Mobile phase: Acetonitrile : 0.05% aqueous trifluoroacetic acid = 1:1

15 Flow rate: 1 ml/min.

Rt = 12.1±0.5

13) Amino acid analysis:

20 Aerothrinic 1 was heated at 120°C in 6N HCl for 24 h, followed by subjecting to amino acid analysis to detect threonine, 3 units of allo-threonine, glycine, alanine, valine, tyrosine, ornithine, 3-hydroxyproline, 4-hydroxyproline, 3-hydroxyglutamine.

Aerothrinic 2

1) Appearance:

25 white solid

2) Molecular weight (FAB-MS method):

m/z 1549 (M+H)⁺

3) Molecular formula:

C₇₁H₁₁₆N₁₄O₂₄

30 4) High resolution mass spectroscopy (for M+H)⁺:

Found: 1549.8384

Calculated for C₇₁H₁₁₇N₁₄O₂₄: 1549.8365

5) UV spectrum (Fig. 5): in methanol:

λ(ε)max (in MeOH): 225±5 (10200 sh), 275±5 (1900), 278±5 (2000)

35 λ(ε)max (in N/10 NaOH-MeOH): 240±5 (7700), 293±5 (2000)

- 25 -

6) IR spectrum (KBr) (Fig. 6):

Main absorption wave numbers (cm^{-1}) are as follows:

3323, 2928, 2856, 1740, 1670, 1531, 1450, 1203, 1137, 837

7) $^1\text{H-NMR}$ spectrum (Fig. 7):

5 400 MHz, in CD_3OD

8) $^{13}\text{C-NMR}$ spectrum (Fig. 8):

100 MHz, in CD_3OD

9) Solubility:

Soluble: water, methanol, dimethylsulfoxide

10) Color reaction:

Positive: ninhydrin, anisaldehyde-sulfuric acid, Iodine vapor, vanillin-sulfuric acid, Rydon-Smith reagent, molybdophosphoric acid

Negative: Sakaguchi reagent, bromocresol green, 2,4-dinitrophenylhydrazine-sulfuric acid

15) 11) Thin-layer chromatography (TLC):

Carrier	Solvent	Rf
Silica gel F254 ¹	<i>n</i> -BuOH: acetone:AcOH:H ₂ O (4:5:1:1)	0.29
	MeOH: H ₂ O (95:5)	0.15

¹ E. Merck AG., Germany

20) 12) High Performance Liquid Chromatography:

Carrier: Capcell Pak C18 gel S120A, 4.6x250 mm (manufactured by Shiseido, Co., LTD.)

Mobile phase: Acetonitrile : 0.05% aqueous trifluoroacetic acid = 1:1

Flow rate: 1 ml/min.

25) Rt = 9.9±0.5

13) Amino acid analysis:

Aerothrinic 2 was heated at 120°C in 6N HCl for 24 h, followed by subjecting to amino acid analysis to detect threonine, 3 units of allo-threonine, glycine, alanine, valine, 3-hydroxytyrosyl (DOPA), ornithine, 3-hydroxyproline, 4-hydroxyproline, 30 3-hydroxyglutamine.

Aerothrinic 3

1) Appearance:

white solid

- 26 -

- 2) Molecular weight (FAB-MS method):
m/z 1533 (M+H)⁺
- 3) Molecular formula:
 $C_{71}H_{116}N_{14}O_{23}$
- 5 4) UV spectrum: in methanol
 $\lambda(\epsilon)_{\text{max}}$ (in MeOH): 225±5 (11000 sh), 275±5 (2000), 280±5 (1900)
 $\lambda(\epsilon)_{\text{max}}$ (in N/10 NaOH-MeOH): 243±5 (7800), 295±5 (1800)
- 5) IR spectrum (KBr):
 Main absorption wave numbers (cm^{-1}) are as follows:
 10 3334, 2928, 2852, 1742, 1662, 1520, 1449, 1202, 1136, 836
- 6) Solubility:
 Soluble: water, methanol, dimethylsulfoxide
- 7) Color reaction:
 Positive: ninhydrin, anisaldehyde-sulfuric acid, Iodine vapor, vanillin-sulfuric acid,
 15 Rydon-Smith reagent, molybdophosphoric acid
 Negative: Sakaguchi reagent, bromocresol green, 2,4-dinitrophenylhydrazine-sulfuric acid
- 8) Thin-layer chromatography (TLC):

Carrier	Solvent	Rf
20 silica gel F254 ^{*1}	<i>n</i> -BuOH: acetone:AcOH:H ₂ O (4:5:1:1)	0.26
	MeOH: H ₂ O (95:5)	0.09
- 9) High Performance Liquid Chromatography:
 Carrier: Capcell Pak C18 gel S120A, 4.6x250 mm (manufactured by Shiseido, Co.,
 25 LTD.)
 Mobile phase: Acetonitrile : 0.05% aqueous trifluoroacetic acid = 1:1
 Flow rate: 1 ml/min.
 Rt = 9.1±0.5
- 10) Amino acid analysis:
 30 Aerothrinic 3 was heated at 120°C in 6N HCl for 24 h, followed by subjecting to amino acid analysis to detect threonine, 3 units of allo-threonine, glycine, alanine, valine, tyrosine, ornithine, 3-hydroxyproline, 4-hydroxyproline, 3-hydroxyglutamine.

- 27 -

Example 1

Preparation of Aerothricin 132

(a) To a mixture of Aerothricin 3 (500 mg, 0.326 mmol) and triethylamine (682 μ l, 4.89 mmol) in MeOH (10 ml) was added acrylonitrile (214 μ l, 3.27 mmol) at room temperature. The mixture was stirred for 20 hours at room temperature. After the solvent was evaporated *in vacuo*, the residue was dissolved in *n*-butanol and washed with diluted hydrochloric acid and water successively. The organic layer was evaporated *in vacuo*. The crude residue was purified by preparative reverse HPLC, the detailed condition of which is shown below. The appropriate fraction were combined, frozen and lyophilized to give 207 mg of Aerothricin 120 as a colorless amorphous solid.

HPLC(Rt) 27.5 min (column F, flow rate: 10 ml/min, eluent: 0.05% Trifluoroacetic acid : 0.05% Trifluoroacetic acid-Acetonitrile = 53:47); FAB-MS (m/z) : 1586 [M+H]⁺.

(b) To a stirred solution of Boc-L-Orn(Boc)-OH (46 mg, 0.138 mmol) in DMF (2 ml) were added BOP reagent (62 mg, 0.14 mmol), HOBT hydrate (22 mg, 0.144 mmol) and N-ethyldiisopropylamine (24 μ l, 0.138 mmol). After being stirred for 2 h at room temperature, a solution of Aerothricin 120 (100 mg, 0.063 mmol) and N-ethyldiisopropylamine (24 μ l, 0.138 mmol) in DMF (2 ml) was added to the reaction mixture. After being stirred for 20 h at room temperature, the solvent was evaporated *in vacuo*.

A solution of the crude residue obtained above in TFA (3 ml) was stirred at 0°C for 30 min. The reaction vessel was opened and TFA was evaporated under a stream of dry nitrogen. The residue was purified by preparative reverse phase HPLC, the detailed condition of which is shown below. The pure fractions were combined, frozen and lyophilized to give 60.5 mg of the compound-1 as a white amorphous solid.

HPLC(Rt) 20.7 min (column F, flow rate: 10 ml/min, eluent: 0.05% Trifluoroacetic acid : 0.05% Trifluoroacetic acid-Acetonitrile = 57:43); FAB-MS (m/z) : 1700 [M+H]⁺.

(c) To a mixture of the compound-1 (60.5 mg, 0.0356 mmol) in dioxane (2 ml) and water (2 ml) was added 10% palladium on charcoal (10 mg), and the reaction vessel was filled with hydrogen. After being stirred for 14 hours at room temperature, the mixture was filtered through membrane filter (pore size : 0.2 mm) and the solvent was evaporated *in vacuo*. The crude residue was purified by preparative reverse HPLC, the detailed condition

- 28 -

of which is shown below. The appropriate fraction were combined, frozen and lyophilized to give 27.8 mg of Aerothrinic 132 as a colorless amorphous solid.

HPLC(Rt) 18.9 min (column F, flow rate: 10 ml/min, eluent: 0.05% Trifluoroacetic acid : 0.05% Trifluoroacetic acid-Acetonitrile = 60:40); FAB-MS (m/z) : 1704 [M+H]⁺.

5

Example 2

Preparation of Aerothrinic 134

- (a) To a stirred solution of Fmoc-L-Orn(Boc)-OH (379 mg, 0.834 mmol) in DMF (6 ml) were added BOP reagent (368 mg, 0.832 mmol), HOBT hydrate (128 mg, 0.836 mmol) and N-ethyldiisopropylamine (145 µl, 0.832 mmol). After being stirred for 2 h at room temperature, a solution of Aerothrinic 120 (600 mg, 0.378 mmol) and N-ethyldiisopropylamine (145 µl, 0.832 mmol) in DMF (3 ml) was added to the reaction mixture. After being stirred for 18 h at room temperature, piperidine (3 ml) was added to the mixture. The reaction mixture was stirred for 10 minutes at room temperature. The solvent was evaporated *in vacuo*. The residue was washed with dichloromethane and diethylether to remove the reagents. The crude product was used for the next step without further purification.

- (b) To a solution of the crude product (320 mg) obtained above in MeOH (10 ml) were added (2-oxo-ethyl)carbamic acid *tert*-butylester (crude, 530 mg), AcOH (2 ml) and NaBH₃CN (210 mg, 3.342 mmol) in MeOH (4 ml). After the mixture was stirred for 20 h at room temperature, the reaction mixture was concentrated *in vacuo*. The residue was dissolved in *n*-butanol and washed with diluted hydrochloric acid and water successively. The organic layer was evaporated *in vacuo*.

- A solution of the crude residue obtained above in TFA (6 ml) was stirred at 0°C for 30 min. The reaction vessel was opened and TFA was evaporated under a stream of dry nitrogen. The residue was purified by preparative reverse HPLC, the detailed condition of which is shown below. The appropriate fraction were combined, frozen and lyophilized to give 88.2 mg of the compound-2 as a white amorphous solid.

- HPLC(Rt) 22.4 min (column F, flow rate: 10 ml/min, eluent: 0.05% Trifluoroacetic acid : 0.05% Trifluoroacetic acid-Acetonitrile = 60:40)

- 29 -

(c) To a mixture of the compound-2 (88.2 mg, 0.0494 mmol) in dioxane (1.5 ml) and water (1.5 ml) was added 10% palladium on charcoal (20 mg), and the reaction vessel was filled with hydrogen. After being stirred for 16 hours at room temperature, the mixture was filtered through membrane filter (pore size : 0.2 mm) and the solvent was evaporated
5 in *vacuo*. The crude residue was purified by preparative reverse HPLC, the detailed condition of which is shown below. The appropriate fraction were combined, frozen and lyophilized to give 22.0 mg of Aerothrin 134 as a colorless amorphous solid.

HPLC(Rt) 25.7 min (column F, flow rate: 10 ml/min, eluent: 0.05% Trifluoroacetic acid : 0.05% Trifluoroacetic acid-Acetonitrile = 63:37); FAB-MS (m/z) : 1790 [M+H]⁺.

10

Example 3

Preparation of Aerothrin 135

Aerothrin 135 was prepared according to the method similar to that described In Example 2:

15 HPLC(Rt) 25.5 min (column F, flow rate: 10 ml/min, eluent: 0.05% Trifluoroacetic acid : 0.05% Trifluoroacetic acid-Acetonitrile = 63:37); FAB-MS (m/z) : 1790 [M+H]⁺.

Example 4

Preparation of Aerothrin 136

20 (a) To a stirred solution of Fmoc-L-Orn(Boc)-OH (379 mg, 0.834 mmol) in DMF (6 ml) were added BOP reagent (368 mg, 0.832 mmol), HOBT hydrate (128 mg, 0.836 mmol) and N-ethyldiisopropylamine (145 µl, 0.832 mmol). After being stirred for 2 h at room temperature, a solution of Aerothrin 120 (600 mg, 0.378 mmol) and N-ethyldiisopropylamine (145 µl, 0.832 mmol) in DMF (3 ml) was added to the reaction
25 mixture. After being stirred for 18 h at room temperature, piperidine (3 ml) was added to the mixture. The reaction mixture was stirred for 10 minutes at room temperature. The solvent was evaporated in *vacuo*. The residue was washed with dichloromethane and diethylether to remove the reagents. The crude product was used for the next step without further purification.

30

- 30 -

(b) To a solution of the crude product (300 mg) obtained above in EeOH (10 ml) were added acrylonitrile (110 μ l, 1.68 mmol) and N-ethyldiisopropylamine (44 μ l, 0.253 mmol). After the mixture was stirred for 14 h at room temperature, acrylonitrile (440 μ l, 6.72 mmol) and N-ethyldiisopropylamine (44 μ l, 0.253 mmol) were added. After the mixture 5 was stirred for 22 h at room temperature, acrylonitrile (220 μ l, 3.36 mmol) and N-ethyldiisopropylamine (44 μ l, 0.253 mmol) were added. After the mixture was stirred for 6 h at room temperature, the reaction mixture was concentrated *in vacuo*. The residue was washed with dichloromethane and diethylether to remove the reagents.

A solution of the crude residue obtained above in TFA (3 ml) was stirred at 0°C for 30 10 min. The reaction vessel was opened and TFA was evaporated under a stream of dry nitrogen. The residue was purified by preparative reverse HPLC, the detailed condition of which is shown below. The appropriate fraction were combined, frozen and lyophilized to give 104.6 mg of the compound-3 as a white amorphous solid.

HPLC(Rt) 27.8 min (column F, flow rate: 10 ml/min, eluent: 0.05% Trifluoroacetic acid : 15 0.05% Trifluoroacetic acid-Acetonitrile = 57:43)

(c) To a mixture of the compound-3 (104.6 mg, 0.0596 mmol) in dioxane (3 ml) and water (3 ml) was added 10% palladium on charcoal (25 mg), and the reaction vessel was filled 20 with hydrogen. After being stirred for 16 hours at room temperature, the mixture was filtered through membrane filter (pore size : 0.2 mm) and the solvent was evaporated *in vacuo*. The crude residue was purified by preparative reverse HPLC, the detailed condition of which is shown below. The appropriate fraction were combined, frozen and lyophilized to give 40.3 mg of Aerothrinic 136 as a colorless amorphous solid.

HPLC(Rt) 25.8 min (column F, flow rate: 10 ml/min, eluent: 0.05% Trifluoroacetic acid : 25 0.05% Trifluoroacetic acid-Acetonitrile = 63:37); FAB-MS (m/z) : 1761 [M+H]⁺.

Example 5

Preparation of Aerothrinic 133

(a) To a solution of Aerothrinic 3 monoTFA salt (2.5g) in DMF (10 ml) was added Fmoc-D-Orn(Boc)OH (850 mg), BOP (180 mg), HOBT (279 mg) and N-ethyldiisopropylamine (0.795 ml). After the mixture being stirred for 3 hrs. at room temperature, piperidine (4.0 ml) was added. The stirring was continued for 30 min. at room temperature, and then the solvent was evaporated *in vacuo*. The residue was dissolved in dichloromethane, and

- 31 -

dropwise addition of ether gave the crude product as white amorphous powder. It was washed with ether and used in the next step without further purification.

(b) The crude product obtained above was dissolved in DMF (20 ml). To this solution was added Boc-L-Orn(Boc)OH (656 mg), BOP (872 mg), HOBT (302 mg) and N-ethyldiisopropylamine (0.793 ml). After the mixture was stirred for 3 hrs. at room temperature, the solvent was evaporated *in vacuo*.

(c) TFA (15 ml) was added at 0° C to the residue obtained above. After the mixture was stirred for 30 min. at 0° C, ether was added dropwise to give a white precipitate. It was washed with ether and purified by preparative reverse phase HPLC. The pure fractions were combined, frozen and lyophilized to give 515 mg of Aerothrin 133 as a colorless amorphous solid:

HPLC(Rt): 19.7 min. (column F, flow rate: 10 ml/min., eluent: 0.05% trifluoroacetic acid-water : 0.05% trifluoroacetic acid-acetonitrile = 60:40); FAB-MS (m/z): 1761 [MH⁺].

15

Example 6

Preparation of Aerothrin 137

(a) To a solution of Aerothrin 3 monoTFA salt (622 mg) in dichloromethane (16 ml) and MeOH (4 ml) was added N-Boc-aminoethanal (120 mg) and N-ethyldiisopropylamine (0.072 ml). After being stirred for 1 hr at room temperature, to the reaction mixture was added sodium cyanoborohydride (48 mg) and sulfuric acid (0.04 ml). After the reaction mixture was stirred for 72 hr at room temperature, the solvent was evaporated *in vacuo* and then 0.1N HCl was added. It was extracted with nBuOH and concentrated.

(b) The residue was dissolved in DMF (6 ml), to which was added 2-(S)-[bis-(2-Boc-aminoethyl)amino]-5-Boc-aminopentanoic acid (294 mg), HOAt (77 mg), HBTU (215 mg) and N-ethyldiisopropylamine (0.148 ml). After being stirred for 48 hr at room temperature, the solvent was evaporated *in vacuo* and the residue was dissolved in dichloromethane. To this solution was added ether to give a white precipitate. It was washed with ether and used in the next step without further purification.

(c) To the compound obtained above was then added TFA (3 ml) at 0°C. After being stirred for 30 min. at 0°C, ether was added to the reaction mixture to give a white precipitate. It was washed with ether and purified by preparative reverse phase HPLC. The pure fractions were combined, frozen and lyophilized to give 95 mg of Aerothrin 137 as a colorless amorphous solid:

- 32 -

HPLC(Rt): 14.6 min. (column F, flow rate: 10 ml/min., eluent: 0.05% trifluoroacetic acid-water : 0.05% trifluoroacetic acid-acetonitrile = 61:39); FAB-MS (m/z): 1776 [M+H]⁺.

5

Example A

Injectable solutions each containing the following ingredients were manufactured in the conventional manner per se:

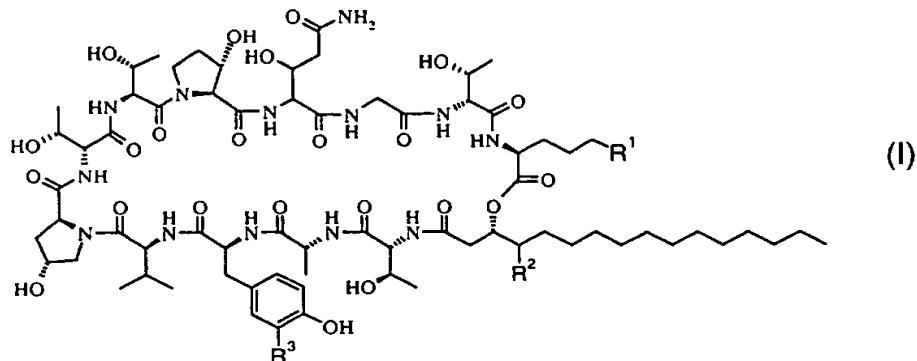
Aerothrin 132	20 mg
di-Sodium hydrogenphosphate, anhydrous	7.6 mg
Sodium diphosphate dihydrate	2.0 mg
Ethyl alcohol	150 mg
Distilled water, deionized, sterile	850 mg
Total	1029.6 mg

- 33 -

ClaimsEPO - Munich
24

17. Jan. 2000

1. Aerothricins represented by the Formula (I),



wherein

R^1 is N-(3-aminopropyl)-N-[(2S)-2,5-diaminovaleryl]amino, N-(3-aminopropyl)-N-[-5-amino-2-[N,N-bis(2-aminoethyl)amino]valeryl]amino, N-(3-aminopropyl)-N-[-5-amino-2-[N-(3-aminopropyl)amino]valeryl]amino, N-(2-aminoethyl)-N-[-5-amino-2-[N,N-bis(2-aminoethyl)amino]valeryl]amino or ornityl-ornitylamino;

R^2 is hydrogen or methyl;

R^3 is hydrogen or hydroxy;

and pharmaceutically acceptable salts thereof.

2. Aerothrin according to Claim 1, wherein R^1 is N-(3-aminopropyl)-N-[(2S)-2,5-diaminovaleryl]amino, N-(3-aminopropyl)-N-[(2S)-5-amino-2-[N,N-bis(2-aminoethyl)amino]valeryl]amino, N-(3-aminopropyl)-N-[(2R)-5-amino-2-[N,N-bis(2-aminoethyl)amino]valeryl]amino, N-(3-aminopropyl)-N-[(2S)-5-amino-2-[N-(3-aminopropyl)amino]valeryl]amino, N-(2-aminoethyl)-N-[(2S)-5-amino-2-[N,N-bis(2-aminoethyl)amino]valeryl]amino or (L)-ornityl-(D)-ornitylamino.
3. Aerothricins according to any of Claims 1 or 2, wherein R^2 and R^3 are hydrogen.
4. Aerothrin according to any of Claims 1 to 3, wherein R^1 is N-(3-aminopropyl)-N-[(2S)-2,5-diaminovaleryl]amino.
5. Aerothrin according to any of Claims 1 to 3, wherein R^1 is (L)-ornityl-(D)-ornitylamino.

- 34 -

6. Aerothrinic according to any of Claims 1 to 3, wherein R¹ is N-(3-aminopropyl)-N-[(2S)-5-amino-2-[N,N-bis(2-aminoethyl)amino]valeryl]amino.
7. Aerothrinic according to any of Claims 1 to 3, wherein R¹ is N-(3-aminopropyl)-N-[(2R)-5-amino-2-[N,N-bis(2-aminoethyl)amino]valeryl]amino.
- 5 8. Aerothrinic according to any of Claims 1 to 3, wherein R¹ is N-(3-aminopropyl)-N-[(2S)-5-amino-2-[N-(3-aminopropyl)amino]valeryl]amino.
9. Aerothrinic according to any of Claims 1 to 3, wherein R¹ is N-(2-aminoethyl)-N-[(2S)-5-amino-2-[N,N-bis(2-aminoethyl)amino]valeryl]amino.
10. A process for the preparation of a compound of Claim 4, which process comprises (a) 10 N-alkylation of a compound of Formula (II) [wherein R¹ is an amino group; R² and R³ are as defined in Claim 1] with acrylonitrile, (b) acylation of the resulting compound with N-protected ornitine, (c) removal of the amino protecting groups (P₁ and P₂) and (d) reduction of the cyano group.
11. A process for the preparation of the compounds of Claims 6 and 7, which process 15 comprises (a) N-alkylation of a compound of Formula (II) [wherein R¹ is an amino group; R² and R³ are as defined in Claim 1] with acrylonitrile, (b) acylation of the resulting compound with N-protected ornitine, (c) removal of the amino protecting group adjacent to the keto-group (P₁), (d) reductive N-alkylation with N-protected amino-acetaldehyde, (e) removal of the remaining amino protecting group (P₂), and (f) 20 reduction of the cyano group.
12. A process for the preparation of a compound of Claim 9, which process comprises (a) reductive N-alkylation of a compound of Formula (II) [wherein R¹ is an amino group; R² and R³ are as defined in Claim 1] with N-protected amino-acetaldehyde, (b) acylation of the resulting compound with N-protected ornitine, (c) removal of the amino protecting group adjacent to the keto-group (P₁), (d) reductive N-alkylation 25 with N-protected amino-acetaldehyde, (e) removal of the remaining amino protecting group (P₂), and (f) reduction of the cyano group.
13. A process for the preparation of a compound of Claim 8, which process comprises (a) N-alkylation of a compound of Formula (II) [wherein R¹ is an amino group; R² and R³ 30 are as defined in Claim 1] with acrylonitrile, (b) acylation of the resulting compound with N-protected ornitine, (c) removal of the amino protecting group adjacent to the keto-group (P₁), (d) N-alkylation with acrylonitrile, (e) removal of the remaining amino protecting group (P₂), and (f) reduction of the cyano group.

- 35 -

14. A process for the preparation of a compound of Claim 5, which process comprises (a) acylation of a compound of Formula (II) [wherein R¹ is an amino group; R² and R³ are as defined in Claim 1] with N-protected ornitine, (b) removal of the amino protecting group adjacent to the keto-group (P₁), (c) acylation with N-protected ornitine, and (d) removal of the remaining amino protecting group (P₂).
5

15. Aerothricins of any of Claim 1 to 9 for the use in medical therapy.

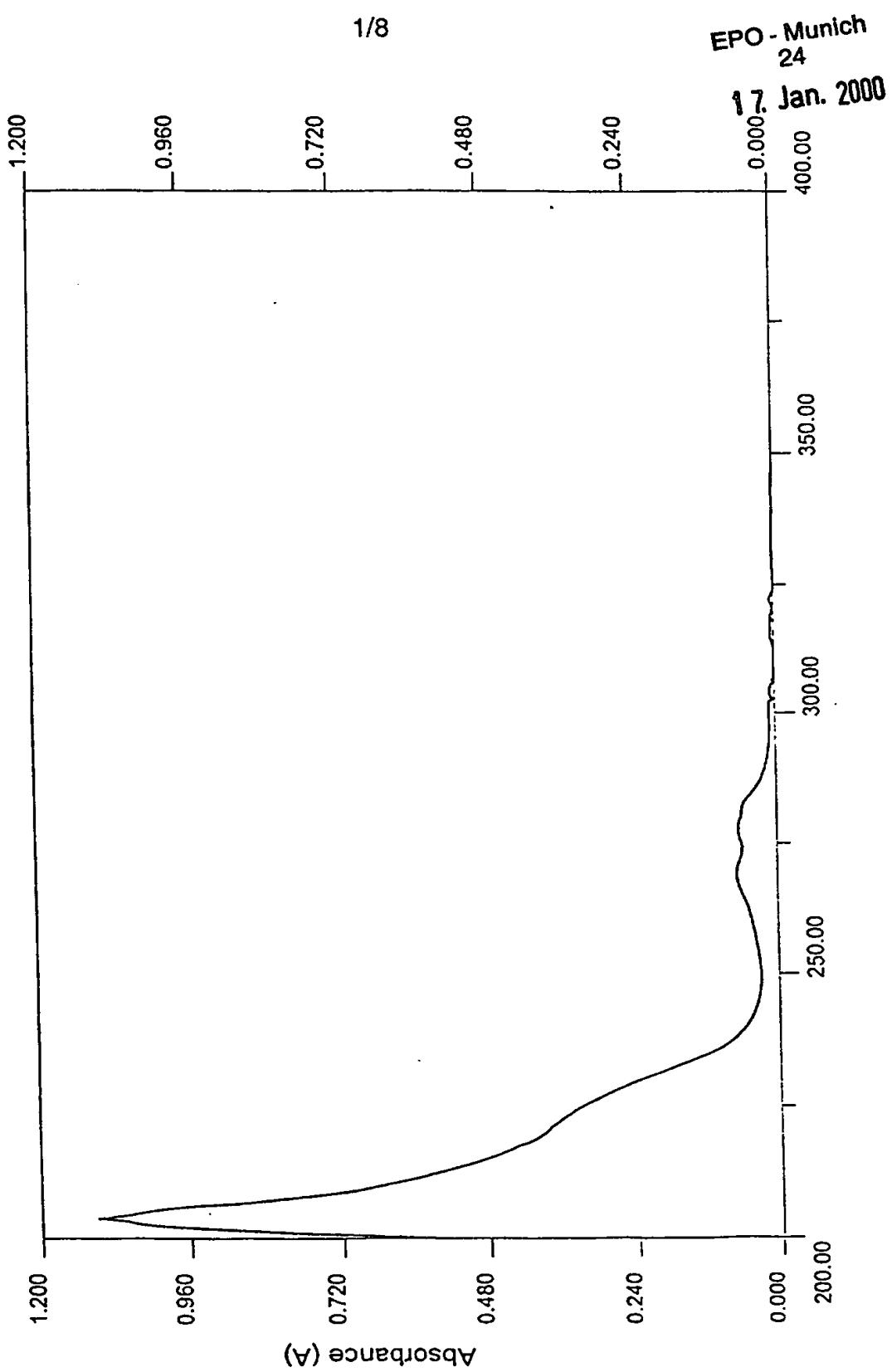
16. A pharmaceutical composition comprising a compound of any of Claims 1 to 9 and a pharmaceutically acceptable carrier.

17. The use of a compound of any of Claims 1 to 9 for the preparation of medicaments
10 comprising a compound of any of Claims 1 to 9 for the treatment or prophylaxis of mycoses.

18. A method for the prophylactic and/or therapeutic treatment of infectious diseases caused by pathogenic microorganisms which comprises administering a compound of any of Claims 1 to 9 to a human being or an animal.

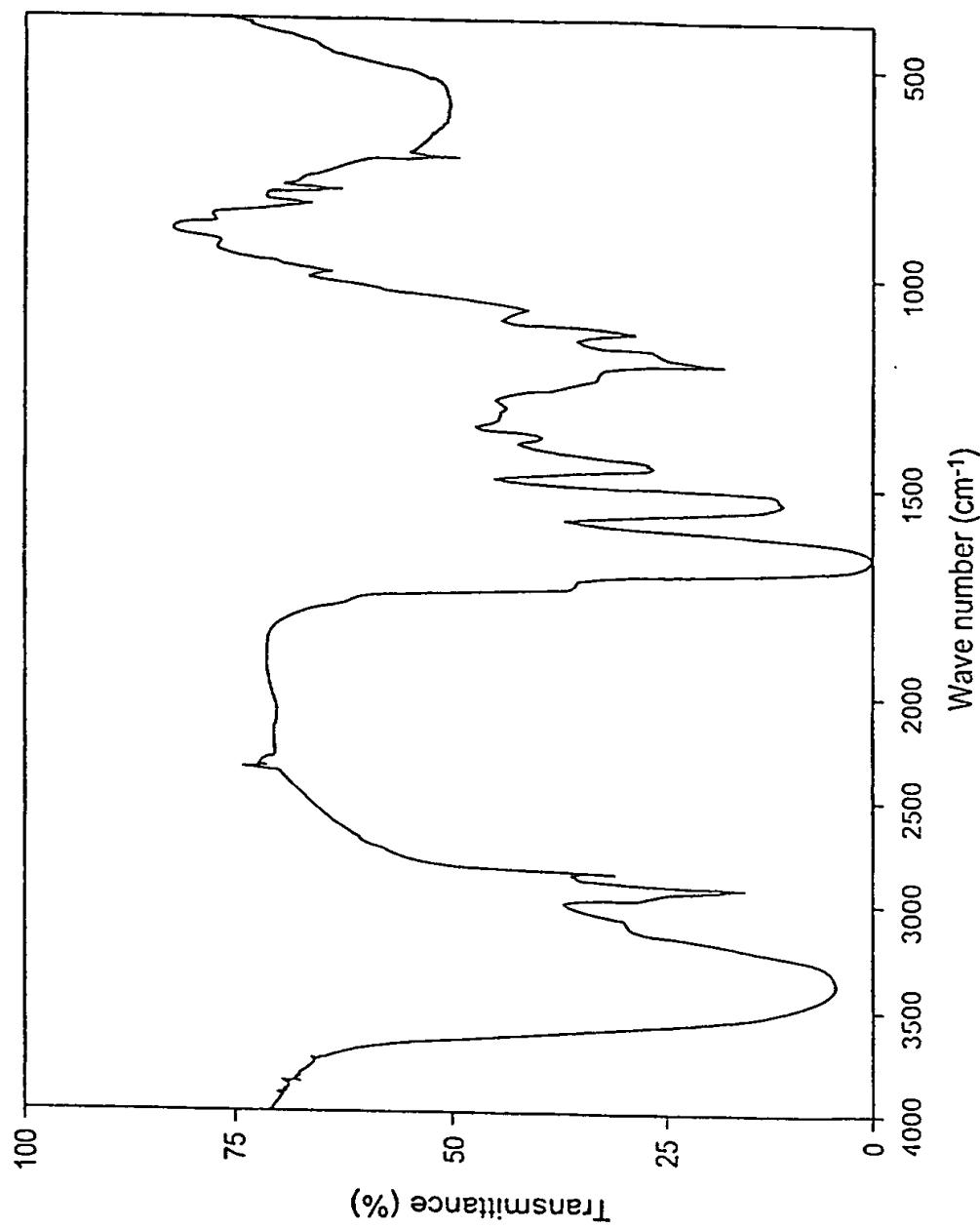
15 19. The invention as described hereinbefore.

Fig. 1



2/8

Fig. 2



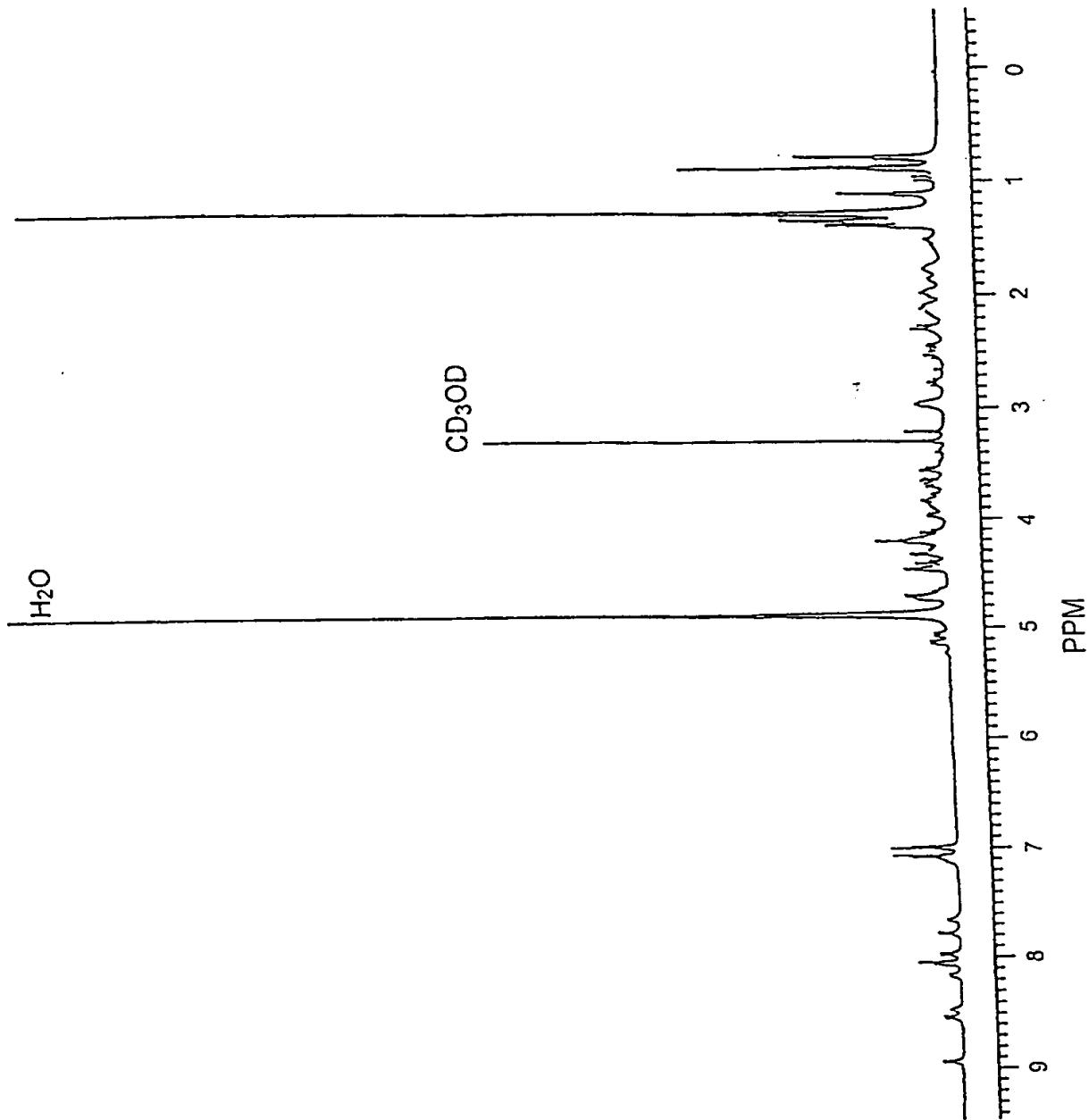
17-01-2000

EP00100807.7

DRAW

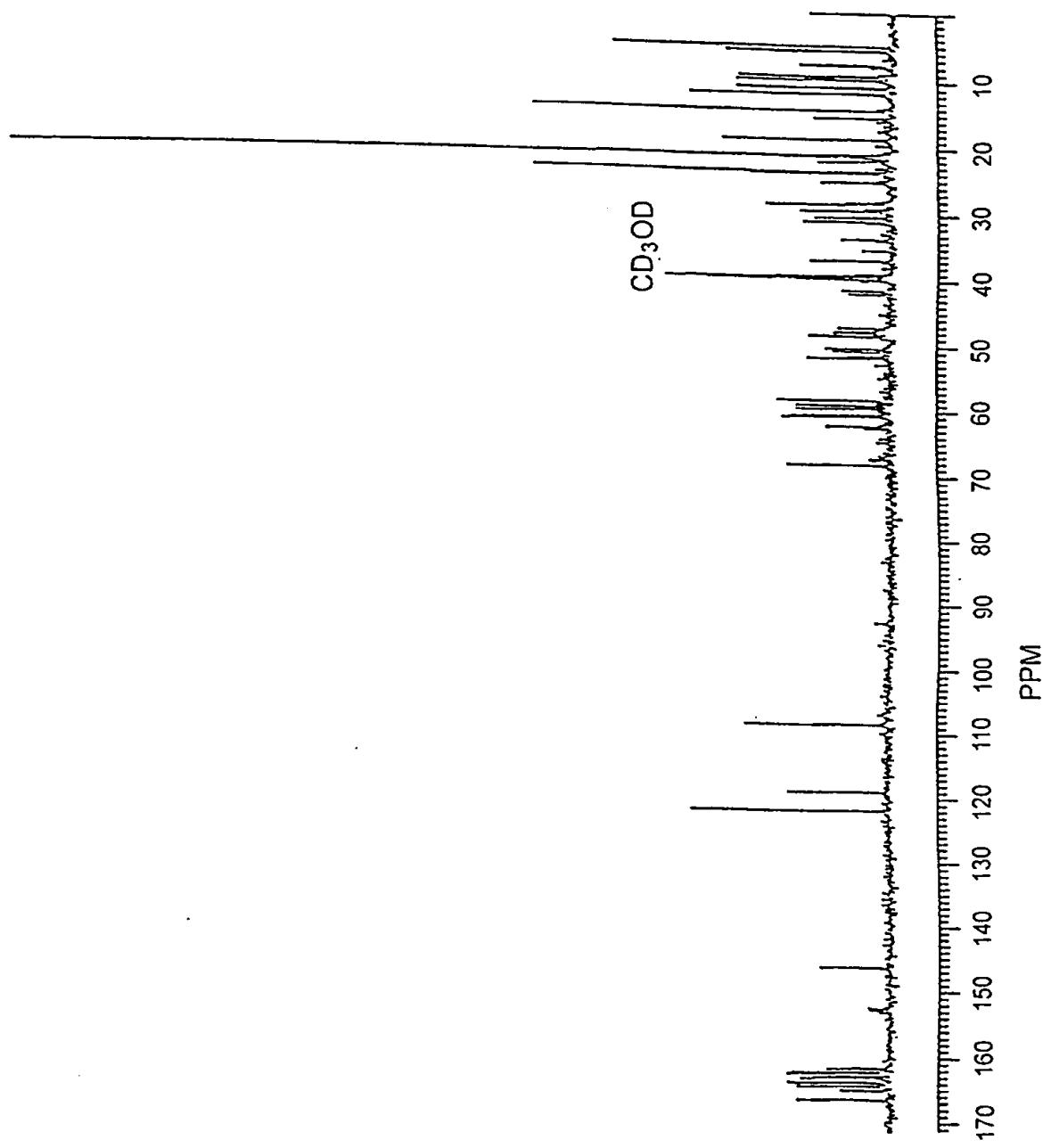
3/8

Fig. 3



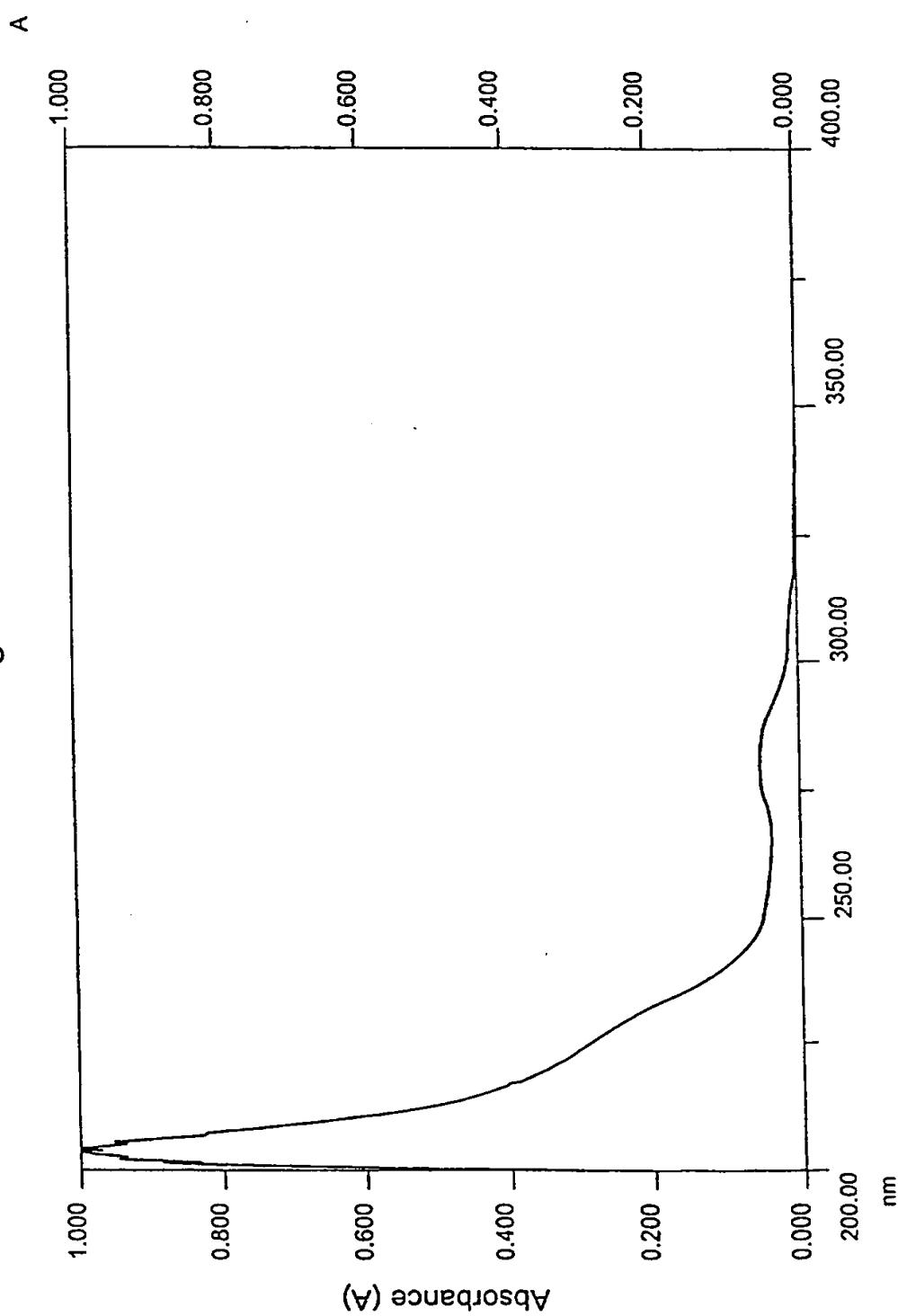
4/8

Fig. 4



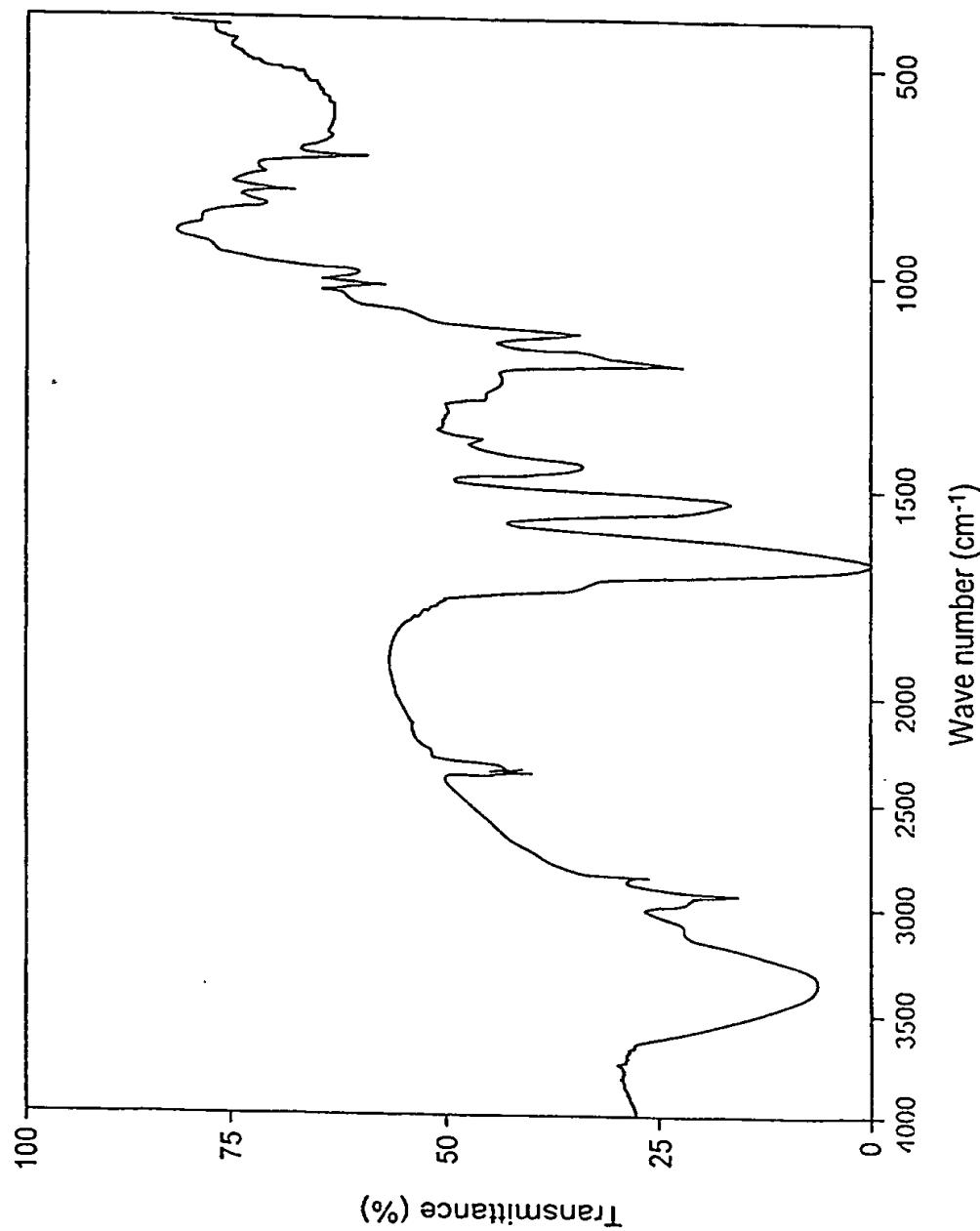
5/8

Fig. 5



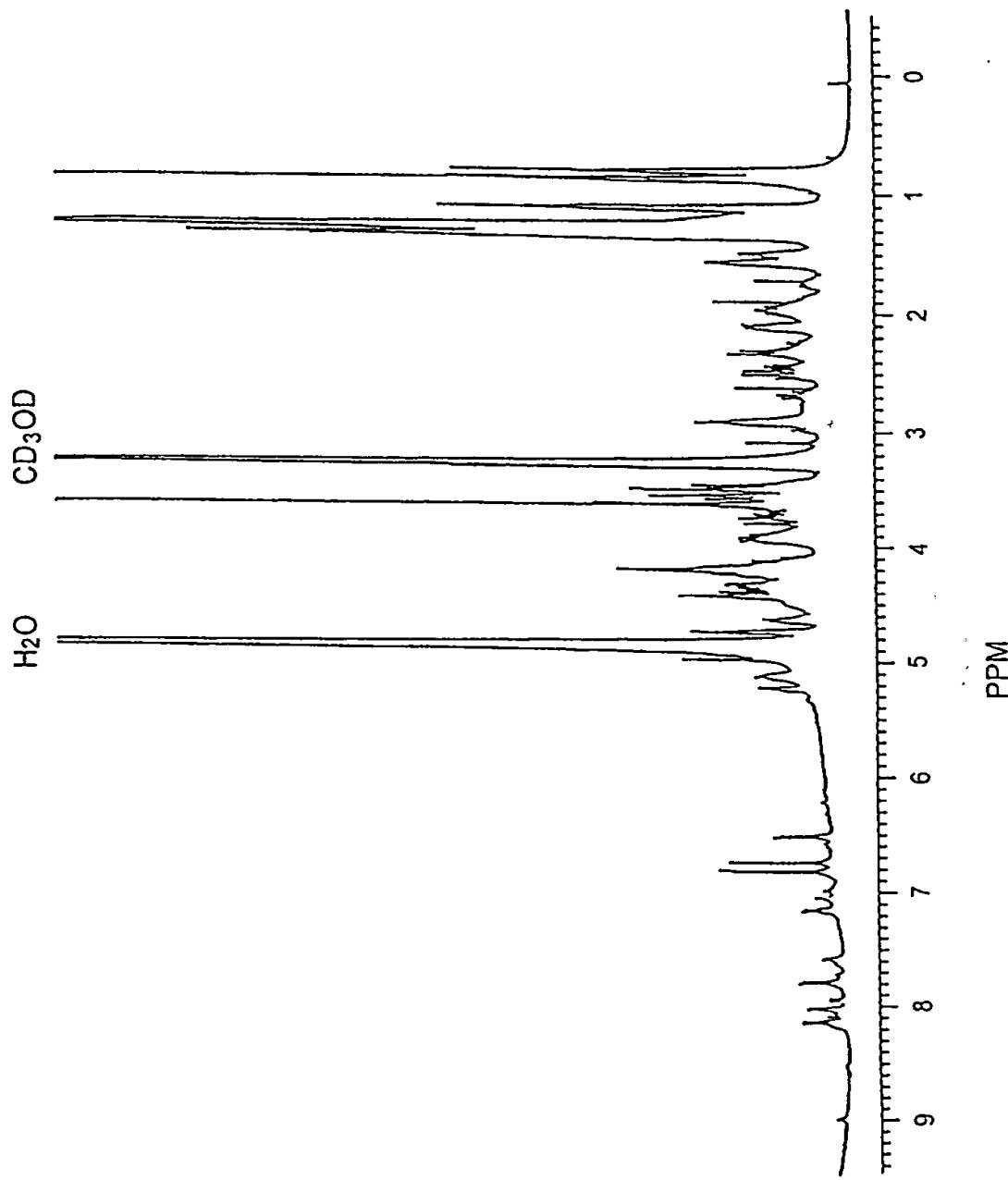
6/8

Fig. 6



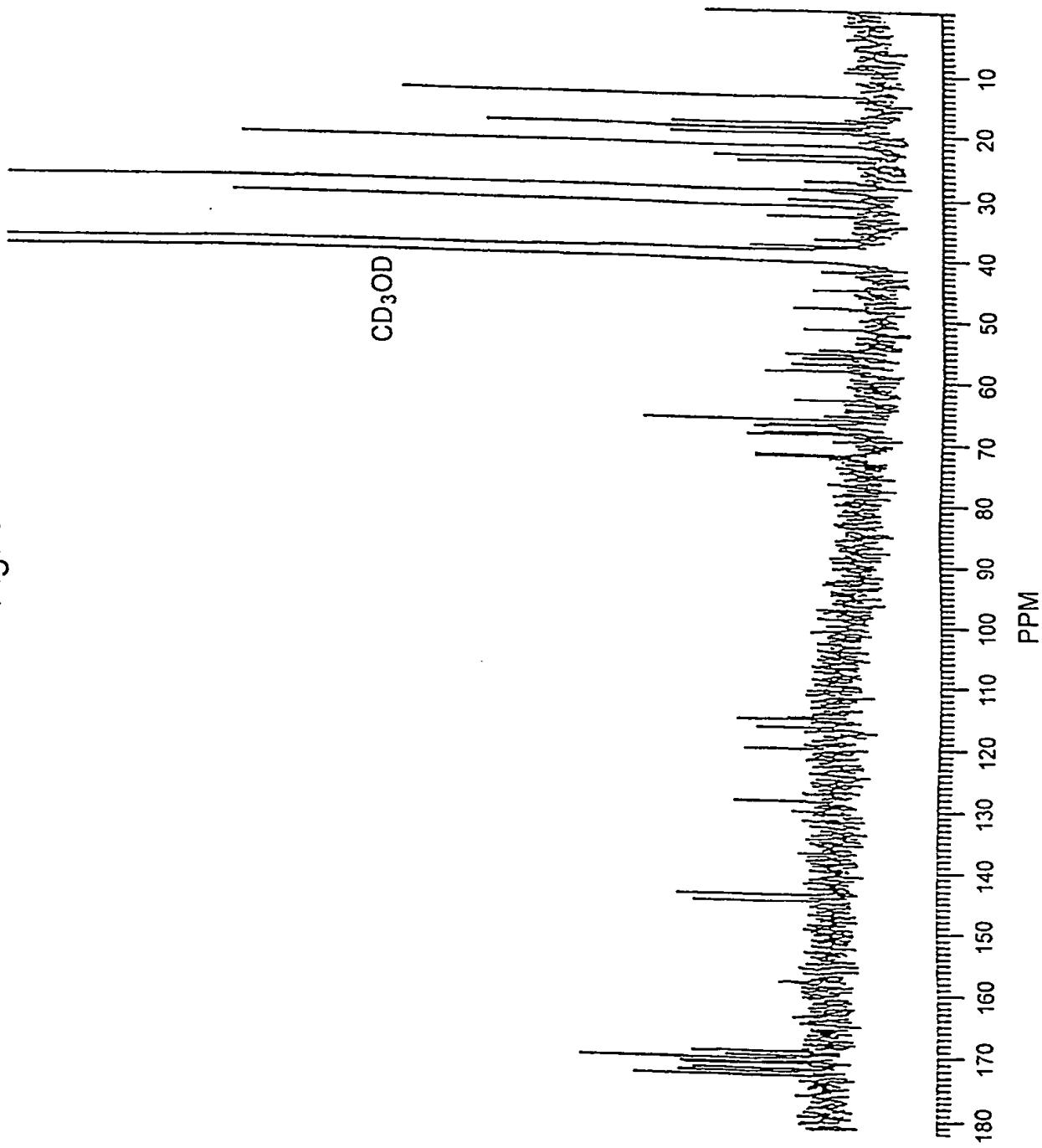
7/8

Fig. 7



8/8

Fig. 8

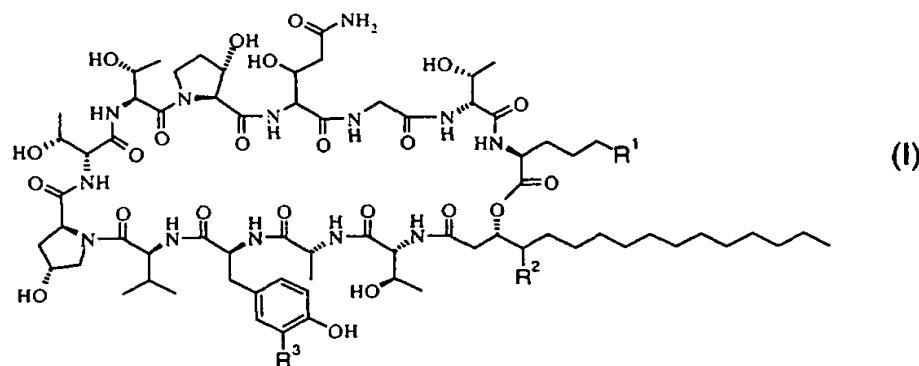


- 36 -

EPO - Munich
24Abstract

17. Jan. 2000

The present invention relates to novel Aerothricins represented by the Formula (I),



wherein R¹, R² and R³ are as defined in Claim 1; and pharmaceutically acceptable salts thereof.

The present invention also relates to a pharmaceutical composition comprising an Aerothrin of the Formula (I) and a pharmaceutically acceptable carrier. Furthermore, the present invention relates to the use of such Aerothricins for the preparation of medicaments, as well as to processes for the preparation of the Aerothricins of the Formula (I).

